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1. Introduction

Food safety has been worldwide recognized as an impact on public health, particularly the microbiological safety of animal-origin food. Major foodborne bacteria diseases encompass campylobacteriosis, enterohaemorrhagic *E.coli* infections, salmonellosis, listeriosis and yersioniosis. Main age group affected are children under 4 years old and persons who older than 50 years old (CDC 2010). The World Health Organization (WHO) reported that for 2005, 1.8 million people died worldwide from diarrheal diseases. A great proportion of these cases can be attributed to contamination of food and drinking water. In the United States of America (USA), around 76 million cases of foodborne diseases, resulting in 325,000 hospitalizations and 5,000 deaths, are estimated to occur each year (WHO 2008).

Many studies on foodborne pathogens among pork or chicken production chains have been performed to provide surveillance data. Compared to chicken and pork, little information of these pathogens is available from the lamb production chain. In 2008, the world sheep meat production was estimated 8,255,295 tons, which is 935,521 tons in European Union and 25,000 tons in Germany (FAOSTAT 2009).

Preharvest control is an important step to decrease the risk of contamination in sheep meat. Surveillance and monitoring of foodborne disease provide crucial information on planning, implementing and evaluating food safety systems. Therefore, appropriate information of major microbiological foodborne at farm level will strengthen the surveillance system of foodborne disease.

Objectives

In this study, the prevalence of 5 foodborne bacteria, including thermophilic *Campylobacter*, *Listeria* spp., *Escherichia coli*, *Salmonella* spp. and *Yersinia enterocolitica* at sheep farm in Brandenburg were examined.

2. Literature

2.1 Thermophilic *Campylobacter*

2.1.1 Mirobiology of thermophilic *Campylobacter*

Campylobacter are Gram-negative bacteria, belonging to the family Campylobacteraceae (Doyle and Beuchat 2007). They are 0.2-0.5 μm wide and 0.5-5 μm long. They appear S-shaped and motile with a characteristic cork-screw motion by means of a single, unsheathed, polar flagellum at one or both ends of the cells. The organism is typically microaerophilic, requiring an O_2 concentration between 3-15%, which is lower than in the atmosphere. They are also capnophilic, with a CO_2 concentration between 3-5% (Holt et al. 1994). Thermophilic *Campylobacter*, including *C.jejuni, C.coli, C.lari* and *C.upsaliensis*, have been frequently involved in human enteric diseases. They grow optimally at 42-43^{o}C (Barros-Velazquez et al. 1999).

2.1.2 Campylobacteriosis in humans

Situation of campylobacteriosis in humans

Foodborne Diseases Active Surveillance Network (FoodNet) indicated the *Campylobacter* incidence from 10 US-States in 2009 as 13.02 per 100,000 population or 6,033 cases per 17,468 laboratory-confirmed cases of infection (CDC 2010). Among all EU and EEA/EFTA countries, the incidence of campylobacteriosis in 2007 was 46.73 per 100,000 population, which was higher than the previous one (39.5 in 2006) (ECDC 2009). In 2007, *Campylobacter* was the second leading cause of foodborne outbreaks (28%) in Austria. *C.jejuni* was the predominant species causing gastroenteritis in

humans (Much et al. 2009). In Germany, the incidence of *Campylobacter* was as high as 78.7 per 100,000 population in 2008. *C.jejuni* was mostly isolated (70.8%), whereas *C.coli* was isolated in 5.6% and *C.lari* in 0.6% of cases. However, 21.5% of the isolates have not been differentiated into *C.jejuni* or *C.coli* (Robert-Koch-Institut 2009). In Japan, the *Campylobacter* incidence was estimated as 237 cases per 100,000 population using the Monte-Carlo simulation model (Kubota et al. 2008).

Transmission of Campylobacter

Among 33 outbreaks during 2001-2006 in Australia, consumption of poultry, unpasteurized milk and salads were associated with *Campylobacter* gastroenteritis (Unicomb et al. 2009). In addition, the investigation of an outbreak from a restaurant in Stockholm showed a significant association of gastrointestinal illness with marinated chicken (de Jong and Ancker 2008). Other animal products, such as raw cow's milk, were reported as a cause of two outbreaks in the Netherlands in 2005 and 2007 (Heuvelink et al. 2009).

Apart from outbreak cases, the vast majority (97%) of sporadic cases was attributed to the food chain. Chicken and cattle were the principal source of *C.jejuni* in humans, whereas wild animals and environmental sources were responsible for just 3% (Wilson et al. 2008). Apart from chicken, lettuce and take away foods were the most important risk factors for sporadic cases in Ireland (Danis et al. 2009).

Clinical manifestation

The most frequently affected age for campylobacteriosis in northeast Scotland was children under 5 years old, simultaneously the incidence in rural areas (234 cases/100,000 population) was higher than in urban areas (151 cases/100,000 population) (Strachan et al. 2009). Patients mostly suffered

from vomiting, diarrhea or bloody diarrhea, dizziness, fever, nausea, tiredness and stomach pain (de Jong and Ancker 2008).

2.1.3 *Campylobacter* in animals

Due to the high prevalence of *Campylobacter* in poultry, poultry has been regarded as one of the most important sources of human *Campylobacter* infections. In United Kingdom, 24 of 25 cloacal swab samples from live chicken were positive. Among these isolates, 80.6% were *C.jejuni* and 19.4% were *C.coli* (Colles et al. 2010). In broiler-chickens, 63% of the flocks were infected with *Campylobacter* spp. *C.jejuni* was the most prevalent species in Dakar, Senegal (Cardinale et al. 2004). At chicken farms in Thailand, *C.jejuni* (42.5%) was the majority species, followed by *C.coli* (39.1%). Predominant species found at slaughterhouses was *C.coli* (72.4%), which was higher than *C.jejuni* (17.2%) (Meeyam et al. 2004). Moreover, chicken carcasses in United Kingdom were 100% (25/25) *Campylobacter* positive, with isolates being identified as *C.jejuni* (62%) and *C.coli* (37.2%). In 0.8% of isolates, a separation of *C.jejuni* and *C.coli* was not performed (Colles et al. 2010). Among poultry products in northern Ireland, high detection rates were recovered from retail (chicken 91%, turkeys 56% and ducks 100%) (Moran et al. 2009).

Campylobacter is a significant cause of sheep abortion. In New Zealand, the leading cause of diagnosed sheep abortion is *C.fetus* subsp. *fetus* and the species *C.jejuni* and *C.coli* have also been implicated (Mannering et al. 2006). However, *C.jejuni* and *C.coli* were also isolated from healthy cattle and sheep in the east of Turkey (Acik and Cetinkaya 2006). The isolation of *Campylobacter* from cattle fecal samples was 22% from northeastern and southwestern Scotland (Rotariu et al. 2009). Furthermore, different products of animal origin in New Zealand indicated that the prevalence of *C.jejuni* and *C.coli* in chicken (89.1%), 9.1% in pork, 3.5% in beef and 10% in unweaned veal (Wong et al. 2007).

Campylobacter in faeces from other animals were reported from rural (northeast and southwest of Scotland) and urban areas (Edinburgh, Glasgow, and Aberdeen): *Campylobacter* was isolated from pigs (26.7%), birds (23.7%), cats (4.5%) and dogs (1.3%) (Ogden et al. 2009). Higher *Campylobacter* detection rates were found in Barbados as followed; dogs (46.9%), cats (37.3%), monkeys (17.1%) and wild birds (39.3%). Among these isolates, *C.jejuni* was the most predominant one, while *C.upsaliensis* and *C.helveticus* were mainly recovered from cats (Workman et al. 2005).

2.1.4 *Campylobacter* along the lamb production chain

At the farm

The shedding rate of *Campylobacter* was lower than the intestinal carriage rate in Lancaster, United Kingdom (Jones et al. 1999). *Campylobacter* was isolated in a percentage of 91.7% from lamb small intestine samples at a slaughterhouse in Lancashire, while the detection rate was lower from faeces collected from grazing sheep (29.3%) (Stanley et al. 1998). The isolation rate of *Campylobacter* spp. in sheep fecal samples was 25% from northeastern and southwestern Scotland (Rotariu et al. 2009). Moreover, *C.jejuni* was the main species (90%) found in grazing sheep in Lancaster (United Kingdom), while *C.coli* was the next one (8%) and *C.lari* made up to 2% (Jones et al. 1999).

At slaughter

Campylobacter spp. was isolated from 114 of 653 (17.5%) slaughtered sheep in Switzerland; 61.3% of sheep farms were positive with *Campylobacter*. The most prevalent species was *C.jejuni* (64.9%), while *C.coli* was identified in 35.1% (Zweifel et al. 2004). In Great Britain, *Campylobacter* obtained from the intestine at slaughterhouse was 43.8% (Milnes et al. 2008). Lairage environmental samples were 5.6% positive with *Campylobacter* in southwest England (Small et al. 2002). In the United States, sheep carcasses during pre-

and postevisceration were found *Campylobacter* positive in 0.5% and 0.6% of cases, respectively (Duffy et al. 2001).

At the market

The isolation rate and the strains of thermophilic *Campylobacter* isolated from retail varied in each of the following studies. The prevalence of *Campylobacter* in Iran from lamb meat was 12.0% (Rahimi et al. 2010). In the United Kingdom, thermophilic *Campylobacter* were isolated from lamb in 7.4% of cases (Little et al. 2008) and 21.6% of samples in Istanbul, Turkey (Bostan et al. 2009). Both, *C.jejuni* and *C.coli*, were identified in 6.9% of lamb and mutton samples in New Zealand (Wong et al. 2007). Sheep liver samples in New Zealand contained a high load of *Campylobacter* (66%, 180/282). Most of the positive samples contained <3 MPN/g of the organism, while only 12 (6.7%) exceeded 100 MPN/g (Cornelius et al. 2005).

2.2 *Listeria* spp.

2.2.1 Microbiology of *Listeria*

Listeria are Gram-positive short rods or sometimes cocci with 0.4-0.5 x 0.5-2 μm. They belong to the family Listeriaceae. The optimum growth temperature is 30-37^{o}C, but they are motile by peritrichous flagella when grown at 20 -25^{o}C (Holt et al. 1994). *L.monocytogenes* is the causative agent of human listeriosis, which is an important foodborne disease. The other species are *L.ivanovii, L.innocua, L.seeligeri, L.welshimeri* and *L.grayi* (Doyle and Beuchat 2007).

2.2.2 Listeriosis in humans

Situation of listeriosis in humans

Listeriosis is a foodborne disease with a high fatality rate (Fretz et al. 2010). In 2009, its incidence was 0.34 per 100,000 population or 158 cases of 17,468 laboratory-confirmed cases of infections from 10 US-states. Of these, the case fatality rate (CFR) was highest in persons aged ≥50 years (17.5%) (CDC 2010). Among all EU and EEA/EFTA countries (Portugal did not report any data), the incidence of listeriosis in 2007 was 0.35 per 100,000 population (ECDC 2009). In Australia during 1997 -2007, *L.monocytogenes* infections were reported in 150 human cases. The average incidence during this period was 0.168 cases per 100,000 population, most of them as systemic infections (Kasper et al. 2009). In Spain, the average incidence of human listeriosis was 0.65 per 100,000 population during 1995-2005 (Garrido et al. 2008). In Germany, the incidence of listeriosis in Germany accounted to 0.4 per 100,000 population in 2008 (Robert-Koch-Institut 2009).

Within *L.monocytogenes*, the predominant serovar found in Austria during 1997-2007 was 4b (54%), followed by 1/2a (31.3%), 1/2b (10%), 1/2c (2.7%), 4d (1.3%) and 3a (0.7%) (Kasper et al. 2009). In Spain, serogroup 4b was predominant (75.8%), followed by 1/2a (18.2%) (Garrido et al. 2008).

Transmission of Listeria

Many products of animal origins were associated with outbreaks of listeriosis. In Austria and Germany 2009, 14 outbreaks have been reported due to consumption of acide curd cheese "Quargel". Among these, 4 cases were lethal (Fretz et al. 2010). Ready-to-eat scalded sausage was identified as the cause of an outbreak in immunocompromised patients at a hospital in the state of Baden-Württemberg in Germany (Winter et al. 2009). Apart from ready-to-eat products, a precooked meal with beef from a meals-on-wheal delivery catering company was the cause of human listeriosis in Denmark (Smith et al.

2010). An outbreak of febrile gastroenteritis in Austria was associated with the consumption of mixed cold-cuts, including jellied pork contaminated with *L.monocytogenes* (Pichler et al. 2009).

Clinical manifestation

L.monocytogenes is the causative agent of human febrile gastroenteritis, with a potential of fatal foodborne infections. Clinical manifestations range from febrile gastroenteritis to more severe invasive forms including sepsis, meningitis, rhomboencephalitis, perinatal infections, and abortions (Allerberger and Wagner 2010). Septicemia (Garrido et al. 2008; Smith et al. 2010) and meningitis were most frequently found in human listeriosis (Garrido et al. 2008).

For listeriosis in Austria, predisposing factors were determined, accounting to age≥ 65 years, pregnancy, carcinoma, blood malignancy, autoimmune disease and status post solid organ transplants (Kasper et al. 2009). Immunocompromised persons are at higher risk than other patients for developing invasive listeriosis with high fatality rates (Winter et al. 2009). Persons ≥ 65 years represented the most affected age group with an incidence of 1.08 per 100,000 population, while the age group under 5 years was found to suffer in a number of 0.49 per 100,000 population, of which 85% were newborns (ECDC 2009).

2.2.3 *Listeria* in animals

Ruminants and poultry are known to be an important source of human listeriosis. The prevalence of *L.monocytogenes* at cattle farms with a history of *L.monocytogenes* infection was 24.4%, which was higher than data from farms without any history (20.2%) from New York state, USA (Nightingale et al. 2004). In northern Spain, *L.monocytogenes* was identified from 46.3% of dairy cattle and 30.6% of beef cattle (Esteban et al. 2009). At slaughterhouse,

the mean prevalence data of *L.monocytogenes* were faeces with 19% (4.8–29%), hides 12% (10–13%), and raw beef products 10% (1.6–24%) (Rhoades et al. 2009). In Jordan, *L.monocytogenes* was found in beef (19.2%), whereas *L.innocua* and *L.welshineri* were identified in 24% and 14.2% of samples, respectively (Awaisheh 2010).

Poultry was most likely to contain *L.monocytogenes* (65.6%, 21/32) in the United Kingdom (MacGowan et al. 1994). The detection rate of *L.monocytogenes* from raw chicken samples in Turkey was 18% (Akpolat et al. 2004). In Jordan, *L.monocytogenes* was found in 15% of poultry, while *L.innocua* and *L.welshineri* were identified in 23% and 15.8% of the samples, respectively (Awaisheh 2010).

L.monocytogenes was isolated in many other animal food products in Turkey, including soft cheese (5%), minced beef (5%) and fish (1%), while none of them was found in sausage samples (Akpolat et al. 2004). High prevalence of *L.monocytogenes* in Italy was found in smoked salmon (10.6%) and in poultry meat samples (8.5%) (Latorre et al. 2007).

2.2.4 *Listeria* along the lamb production chain

At the farm

L.monocytogenes was isolated from healthy sheep and their environment in Iceland (Gudmundsdottir et al. 2004). In US state New York, the prevalence of *L.monocytogenes* was 32.9% in small ruminant farms (sheep and goat), while their prevalence at farms without history of listeriosis was 5.9% (Nightingale et al. 2004). The serovars in sheep farms in Iceland was 1/2a, 1/2b, or 4b (Gudmundsdottir et al. 2004). Of 248 apparently healthy sheep in India, 7 were positive with *L.monocytogenes* and 9 with *L.innocua* (Yadav and Roy 2009).

L.monocytogenes was also identified as a causative agent for abortion in Danish sheep (Agerholm et al. 2006). The outbreak of listeriosis in sheep was caused by feeding contaminated grass silage in Austria. Infected animals developed abortive, encephalitic and septicaemic types of listeriosis (Wagner et al. 2005). Moreover, encephalitic listeriosis was the most frequent cause of Central Nervous System (CNS) lesions. Consequently, the type of samples collected in each study may affect the estimation of prevalence (Oevermann et al. 2008; Oevermann et al. 2010).

At slaughter

In Brazil, fecal samples collected at a slaughterhouse yielded *L.welshimeri* (20%) and *L innocua* (8.6%). Carcass samples carried *L.innocua* (34.8%), *L.monocytogenes* (4.3%), and *L.ivanovii* (1.5%) (Antoniollo et al. 2003). In Turkey, *L.monocytogenes* was found in sheep carcass samples in 5% (8/160) of samples (Akpolat et al. 2004).

At the market

At retail in Brisbane, Australia, lamb was found positive in 40% (20/50) with *Listeria* spp., consisting of 16% *L.monocytogenes* and 24% *L.innocua* (Ibrahim and Mac Rae 1991). A high percentage of lamb (40%, 8/20) contained *L.monocytogenes* (MacGowan et al. 1994), while *L monocytogenes* was not detected in lamb meat samples (Cohen et al. 2006).

2.3 *Escherichia coli*

2.3.1 Microbiology of *Escherichia coli*

Escherichia coli are Gram-negative bacteria belonging to the family Enterobacteriaceae. They are straight rods bacteria (1.1-1.5 x 2.0-6.0 μm), motile by peritrichous flagella or non-motile. The optimal growth temperature

is 37°C (Holt et al. 1994). *E.coli* causing diarrhea in humans is categorized in pathotypes based on virulence properties, mechanism of pathogenicity, clinical syndromes and distinct O:H serotypes. These categories include enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *Escherichia coli* (ETEC), enteroinvasive *Escherichia coli* (EIEC), diffuse-adhering *Escherichia coli* (DAEC), enteroaggregative *Escherichia coli* (EAEC) and enterohaemorrhagic *Escherichia coli* (EHEC). Due to frequency and severity of illness, EHEC is the most significant group among *E.coli* regarding the foodborn aspect. *E.coli* organisms of many different serotypes may produce Shiga-toxins (STEC) (Doyle and Beuchat 2007).

2.3.2 *Escherichia coli* infections in humans

Situation of Escherichia coli infection in humans

Diarrheagenic *Escherichia coli* have been reported in many regions as described in Table 2.1. Most affected age group was children <4 years old. Among this group, the incidence for STEC O157 was 3.84 per 100,000 population, while STEC non-O157 was 2.72 per 100,000 population. The percentage of hospitalized persons was highest among those aged ≥50 years for STEC O157 (59.4%), STEC non-O157 accounted to 34.2%. Case Fatality Rate (CFR) was highest in persons aged ≥50 years for STEC non-O157 (2.6%) and STEC O157 (1.5%) (CDC 2010).

Table 2.1: Incidence of *E.coli* reported from several regions

year	regions	*E.coli*	incidence per 100,000 population	references
2004	New Mexico	STEC	0.9	(Lathrop et al. 2009)
2007	New Mexico	STEC	1.7	(Lathrop et al. 2009)
2008	Germany	*E.coli* enteritis	8.5	(Robert-Koch-Institut 2009)
2009	10 US-states	STEC O157	0.99	(CDC 2010)
2009	10 US-states	STEC non-O157	0.57	(CDC 2010)

Pathotypes of Escherichia coli found in children

Diarrheal disease is a worldwide public health problem, mostly affecting children in developing countries. The prevalence of diarrheagenic *E.coli* has been investigated in studies as described in Table 2.2.

Table 2.2: Prevalence of *E.coli* pathotypes isolated from children with diarrhea

year	regions	*E.coli*	prevalence (%)	references
1996-2000	Thailand	EAEC	10.2	(Ratchtrachenchai et al. 2004)
		EPEC	3.2	
		ETEC	3.0	
		EIEC	0.5	
		STEC	0.04	
2001-2002	Vietnam	EAEC	11.6	(Nguyen et al. 2005).
		EPEC	6.6	
		ETEC	2.2	
		EIEC	2.0	
2005-2006	León, Nicaragua	EAEC	27.8	(Vilchez et al. 2009)
		EPEC	16.0	
		EHEC	2.1	
		EIEC	0.8	

Transmission of Escherichia coli

A wide range of foods and environmental conditions have been recognized as a source of diarrheagenic *E.coli* infections. Particularly ruminants, including sheep and cattle carrying the "activatable *Stx2d* gene", are important reservoirs of human infection (Tasara et al. 2008). Hence, the respective food products are also a vehicle for transmission, for example STEC in semihard and hard raw milk cheese (Zweifel et al. 2010). Consumption of raw milk has been found to be of high risk for *E. coli* O157:H7 infections (CDC 2008; Denny et al. 2008). Fermented sausage containing sheep meat was reported as a source of an STEC O103:H25 outbreak in Norway (Sekse et al. 2009), while

fermented sausage containing beef was the cause of an STEC O26:H11 outbreak in Denmark (Ethelberg et al. 2009).

Other probable sources of infection have been described. VTEC O157 infection was associated with ready-to-eat food, such as lemon-and-coriander chicken wraps (Whittaker et al. 2009), and many reports indicate that some vegetables were associated with outbreaks, such as spinach (Wendel et al. 2009) or lettuce (Friesema et al. 2008).

2.3.3 *Escherichia coli* in animals

Ruminants have been identified as an important reservoir of *Escherichia coli*. The intestinal carriage of VTEC O157 in cattle was 4.7% in Great Britain (Milnes et al. 2008), and the prevalence of *E.coli* O157 at slaughterhouse level was 27.2% at lairage and 28.8% on cow hides (Small et al. 2002). In northern Spain, the herd prevalence of *E.coli* O157:H7 amounted to 7.0% and 1.6% in dairy and beef cattle, respectively, while the prevalence of non *E.coli* O157:H7 in dairy and beef cattle was 20.7% and 46.0% (Oporto et al. 2008). In beef products in Australia, *E.coli* was detected in 17.8% of chill ground beef samples at retail outlet, of which 0.3% were *E.coli* O157 (Phillips et al. 2008). In addition, beef was contaminated with *E.coli* O157:H7 (8%) at retail markets in Ethiopia (Hiko et al. 2008) and 2.2 % of cow milk in Greece contained *E.coli* O157 (Solomakos et al. 2009).

Similar to cattle, goat was also found to contain pathogenic *E.coli*. At a goat slaughterhouse in Ethiopia, *E.coli* O157:H7 was identified in 3.3% of fecal samples, 10.0% of skin swabs, 5.0% of carcasses before wash and 8.3% of carcasses after wash (Mersha et al. 2010). This organism was isolated from the respective products, including 2% *E.coli* O157:H7 in goat meat in Ethiopia (Hiko et al. 2008) and 0.7% (*E.coli* O157) in goat milk in Greece (Solomakos et al. 2009).

In pigs, a low intestinal carriage of VTEC O157 was observed (0.3%) in Great Britain (Milnes et al. 2008), while another study in Spain found neither *E.coli* O157:H7 nor non-*E.coli* O157:H7 (Oporto et al. 2008).

2.3.4 *Escherichia coli* along the lamb production chain

At the farm

Many prevalence studies of *E.coli* at sheep farms have been performed. In Spain, the herd prevalence of *E.coli* O157:H7 was 8.7%, the individual prevalence was 7.3% (Oporto et al. 2008). Shedding of *E.coli* O157 was investigated among sheep during winter in northeast Scotland. The individual shedding prevalence was 5.8%, while the prevalence at farm level was 42.8% (Solecki et al. 2009). In the southwestern United States, fecal and wool samples carried *E.coli* O157:H7 in 9% and 18% of cases, respectively (Edrington et al. 2009).

At slaughter

At slaughterhouse, samples directly collected from animals, carcasses and environment were examined in many studies (Table 2.3). *E.coli* O157:H7 was most frequently found among carcasses before washing (9.8%) in Ethiopia (Mersha et al. 2010) , whereas non-O157 STEC were predominant from pelts in the United States (86.2%) (Kalchayanand et al. 2007).

At the market

Boneless meat samples in Australia were in 24.5% positive with *E.coli*, of which 1.3% were *E.coli* O157:H7 (Phillips et al. 2001). Another product, "chill dices lamb", was *E.coli* positive in 16.7%. Of these isolates, 0.6% were *E.coli* O157 (Phillips et al. 2008). The overall prevalence of *E.coli* O157:H7 from lamb and mutton was 2.5% from Ethiopia (Hiko et al. 2008).

Virulent genes of *E.coli* were examined along the lamb production chain in Burgos, Spain. *E.coli* carrying all three virulence genes (*stx1*, *stx2* and *eae*) were the most prevalent ones in slaughterhouses (69%), whereas *E. coli* with the *eae* gene alone was found more frequently in processing plants (32%), and *stx(1)*- and *stx(2)*-positive *E. coli* were predominant in butcheries in Spain (9-10%) (Osés et al. 2010).

Table 2.3: Prevalence of *E.coli* found in samples from sheep abbatoirs

	samples	*E.coli*	prevalence (%)	regions	references
environment	sheep lairage	*E.coli* O157	2.2	Southwest England	(Small et al. 2002)
animals	fecal samples	*E.coli* O157:H7	5.4	Ethiopia	(Mersha et al. 2010)
	cecum samples	STEC	29	Switzerland	(Zweifel et al. 2004)
	sheep intestine	VTEC O157	0.7	Great Britain	(Milnes et al. 2008)
	fleece	*E.coli* O157	5.5	Southwest England	(Small et al. 2002)
	fleece	*E.coli* O157	5.8	Ireland	(Lenahan et al. 2007)
	fleece	*E.coli* O157	12.8	United States	(Kalchayanand et al. 2007)
	pelts	non-O157 STEC	86.2	United States	(Kalchayanand et al. 2007)
	skin swabs	*E.coli* O157:H7	8.0	Ethiopia	(Mersha et al. 2010)
carcasses	carcasses before washing	*E.coli* O157:H7	9.8	Ethiopia	(Mersha et al. 2010)
	carcasses after washing	*E.coli* O157:H7	8.9	Ethiopia	(Mersha et al. 2010)
	carcass swabs at prechill	*E.coli* O157:H7	1.5	Ireland	(Lenahan et al. 2007)
	carcass swabs at postchill	*E.coli* O157:H7	1	Ireland	(Lenahan et al. 2007)
	preevisceration carcasses	*E.coli* O157:H7	1.6	United States	(Kalchayanand et al. 2007)
	postintervention carcasses	*E.coli* O157:H7	2.9	United States	(Kalchayanand et al. 2007)
	preevisceration carcasses	non-O157 STEC	78.6	United States	(Kalchayanand et al. 2007)
	postintervention carcasses	non-O157 STEC	81.6	United States	(Kalchayanand et al. 2007)

2.4 *Salmonella* spp.

2.4.1 Microbiology of *Salmonella*

Salmonella spp. are Gram-negative facultative anaerobic bacteria belonging to the family Enterobacteriaceae. They are 0.7-1.5 x 2.5 μm straight rods bacteria, usually motile by peritrichous flagella. The optimal growth temperature is 37°C. *Salmonella* are chemoorganotrophic, having both a respiratory and fermentative type of metabolism (Holt et al. 1994). Currently, more than 2,500 serotypes of *Salmonella* have been identified (Foley and Lynne 2008).

2.4.2 Salmonellosis in humans

Situation of Salmonellosis in humans

Annual reports from many regions describe the incidence of human salmonellosis for every year. From 10 US states, FoodNet indicated a salmonellosis incidence in 2009 as 15.19 per 100,000 population or 7,039 cases per 17,468 laboratory-confirmed cases of infection. Before 2004, this incidence was notably decreasing and constant after 2004 (CDC 2010). The European Center for Disease Prevention and Control (ECDC) revealed the incidence in 2007 as 34.26 per 100,000 population by all EU and EEA/EFTA countries, case reports of salmonellosis showed a statistically significant decrease since 2004 (ECDC 2009). Moreover, the incidence of salmonellosis in Germany was 52.2 per 100,000 population in 2008, which decreased from that level during 2002-2007, the median of incidence was 67.4 (Robert-Koch-Institut 2009). Since *Salmonella* infection has been considered as a global disease in both developing and developed countries, the global incidence was estimated 93.8 million cases (5th to 95th percentile, 61.8-131.6 millions) (Majowicz et al. 2010).

Serotypes of Salmonella found in humans

Serotypes of *Salmonella* predominantly found in humans were different as per year and region (Table 2.4).

Table 2.4: Prevalence of *Salmonella* serotype found in humans

year	regions	serotypes of *Salmonella*	prevalence (%)	references
2007	EU and EEA/EFTA	Enteritidis	64.5	(ECDC 2009)
		Typhimurium	16.5	
		Infantis	1.0	
		Newport	0.6	
	Vietnam	Typhimurium	37.5	(Vo et al. 2006)
		Enteriridis	12.5	
		Weltevreden	7.14	
	Germany	Enteritidis	62	(Robert-Koch-Institut 2009)
		Typhimurium	30	
2008	Thailand	Enteritidis	18.6	(NIH 2008)
		Choleraesuis	9.6	
		Stanley	8.9	
		Weltevreden	7.1	
		Typhimurium	6.8	
		Rissen	6.1	
		I. 4,5,12:i:-	5.9	
		Anatum	4.4	
		Corvallis	3.5	
		Kedougou	2.5	
2009	United States	Enteritidis	19.2	(CDC 2010)
		Typhimurium	16.1	
		Newport	12.1	
		Javiana	8.5	
		Heidelberg	3.6	
		Montevideo	3.2	
		I4,[5],12:i:-	3.1	
		Muenchen	2.7	
		Saintpaul	2.5	
		Oranienburg	2.1	

Serotypes found in sporadic outbreaks were different in each source and region. For example, S.Brandenburg was isolated from persons who had contact with sheep in New Zealand (Baker et al. 2007), while S.Enteritidis was the cause of an outbreak from egg-consumption among military in Turkey (Kilic et al. 2010). Marlin mousse, consisting of raw eggs, was identified as the cause of an outbreak in Mauritius (Issack et al. 2009).

Clinical manifestation

Salmonella infection causes gastroenteritis illness. As a consequence, affected patients developed diarrhea, abdominal pain, fever, nausea, headache and vomit (Giraudon et al. 2009; Issack et al. 2009; van Cauteren et al. 2009; Kilic et al. 2010). The incidence of human salmonellosis was highest among children under 5 years, whereas the percentage of patients hospitalized was highest among persons ≥ 50 years old (CDC 2010). From children, particularly infants or newborns, a nosocomial outbreak of S.Typhimurium in Turkey has been reported. Of 22 infants who were infected, two died. These infants developed diarrhea, septicemia, and meningitis (Anil et al. 2009).

2.4.3 *Salmonella* in animals

Since consumption of *Salmonella*-contaminated products, such as meat, poultry, eggs, milk, seafood and fresh produce, is associated with foodborne bacterial illness, food animals are considered as an important source of human salmonellosis (Foley et al. 2008). Particularly, *Salmonella* contaminated poultry is considered to be an important source of human salmonellosis (Hanning et al. 2009).

The prevalence of *Salmonella* found in ruminants was apparently different in each study, depending on study areas and sample types. In Great Britain, *Salmonella* intestinal carriage was 1.4% in cattle (Milnes et al. 2008). The average prevalence was calculated from multiple survey studies. *Salmonella*

was isolated from faeces in 2.9% (0.0-5.5%), hides in 60% (15-71%), chilled carcasses in 1.3% (0.2-6.0%) and raw beef product in 3.8% (0.0-7.5%) (Rhoades et al. 2009). In Ireland during 2002-2004, raw beef and beef products were 0.16% *Salmonella* positive, while *Salmonella* was not isolated from cooked products (Jordan et al. 2006). In contrast, a study from Thailand showed high prevalence in beef at the markets (82%, 41/50) in 2003 (Vindigni et al. 2007). The most common serovars from cattle in Great Britain were S.Typhimurium, S.Mbandaka and S.Dublin (Milnes et al. 2008), whereas a study in Thailand described as predominant serovars found in beef S.Anatum, S.Senftenberg and S.Weltevreden (Vindigni et al. 2007).

In Great Britain, the prevalence of *Salmonella* in pigs was 23.4% (in intestinal carriage), where S.Typhimurium and S.Derby were the most common serovars. Among S.Typhimurium in pigs, serovar DT104 and DT193 were frequently isolated (Milnes et al. 2008). The prevalence of *Salmonella* in raw pork and raw pork products was 2.1% in Ireland, while they were not found in cooked pork and their products (Jordan et al. 2006). S.Typhimurium and S.Derby were frequently isolated in Ireland (Jordan et al. 2006).

High contamination of *Salmonella* in Spain was obtained in retail chicken carcasses (the average detection rate was 49%). The highest contaminated part was skin (50%), while the lowest was Hamburgers from these animals (20%). S.Enteritidis was predominantly isolated (Capita et al. 2003). In raw chicken meat *Salmonella* was found in 2.8% of samples in Ireland, whereas cooked products from there indicated a number of 0.2%. The most predominant serovar were S.Kentucky and S.Typhimurium (Jordan et al. 2006). *Salmonella* was also isolated from 1.4% of raw duck meat in Ireland (Jordan et al. 2006).

Besides food animals, *Salmonella* was also identified in other animal species. Reptiles have been recognized as one source of *Salmonella* infection, for example exposure to pet turtles and aquatic frogs was associated with human S.Typhimurium infections in the United States in 2008 (CDC 2010; CDC 2010). In addition, S.Typhimurium (Phage type DT40 and 56/variant) were

isolated and caused death in some garden and wild birds in Scotland and Great Britain, such as finches and house sparrows (Pennycott et al. 2006; Pennycott et al. 2010). However, wild bird isolates from northern England may not represent a large zoonotic risk (Hughes et al. 2008).

2.4.4 *Salmonella* along the lamb production chain

At the farm

Salmonella was contaminated in 7% of sheep fecal samples in the southwestern United States (Edrington et al. 2009), whereas *Salmonella* contaminated pelts differed from 7.8% (Small et al. 2002) to 50% (Edrington et al. 2009). The *Salmonella* intestinal carriage prevalence in abattoirs in Great Britain was 1.1% in 2002, which indicated an increase compared to a prevalence of 0.1% during 1999-2000. The most common serovar was S.Diarizonae 61:k:1,5,7 and S.Dublin (Milnes et al. 2008).

Waste samples from farms (directly collected from the stall) and stored waste samples (transported from the location of deposition to a secondary store such as a slurry lagoon) were examined. *Salmonella* accounted to an amount of 8.3% and 11% in fresh and stored waste samples, respectively (Hutchison et al. 2005).

At slaughter

The prevalence of *Salmonella* at lairage was different depending on the season. The average isolation rate ranged from 1.1% (Small et al. 2002) to 6.5% (Small et al. 2006). A heavy contamination with S.Typhimurium DT120 in the north of England was observed in environmental samples at an abattoir (59%) during November 1999-April 2000. After that, there was a remarkable reduction in the recovery of the organism and it almost disappeared in August 2000 (Purvis et al. 2005).

Sheep carcasses were *Salmonella* positive at the slaughterhouse at a rate of 0.1% (Phillips et al. 2001), 1.5% (Duffy et al. 2001), 5.7% (Vanderlinde et al. 1999) and 9.6% (Small et al. 2006). Along commercial processing plants in the United States, *Salmonella* isolation rates of 14.4%, 4.3%, and 1.8% for pelts, preevisceration carcasses and postintervention carcasses were obtained. The most frequently identified serotype was S.Heidelberg (Kalchayanand et al. 2007). *Salmonella enterica* subsp. *diarizona* was isolated from 72 of 653 (11%) slaughtered sheep in Switzerland and the strains were serotyped as serovar 61:k:1,5,(7). Animals from 20 of 31 farms of origin were found to be positive for *Salmonella enterica* subsp. *diarizona* (64.5%) (Zweifel et al. 2004).

At the site of a sheep meat plant in Australia, *Salmonella* was detected in 1.3% of boneless meat samples (Phillips et al. 2001), while they were found in 6.5% of frozen export samples (Vanderlinde et al. 1999).

At the market

Salmonella was identified from raw sheep meat and raw meat products (0.2%, 16/6794), whereas none of them was isolated from cooked meat and cooked meat products in Ireland (Jordan et al. 2006). Moreover, diced lamb samples in Australia were found to be *Salmonella* positive at a rate of 0.6% (2/360), with S.Infantis and S.Typhimurium as serotypes (Phillips et al. 2008).

2.5 *Yersinia enterocolitica*

2.5.1 Microbiology of *Yersinia enterocolitica*

The genus *Yersinia* belongs to the family Enterobacteriaceae (Doyle and Beuchat 2007). They are Gram-negative straight rods with 0.5-0.8 x 1-3 μm, sometimes approaching a spherical shape. The optimal growth temperature is 28-30^{o}C. This organism is nonmotile, but they are motile when grown below

30°C (Holt et al. 1994). The four known pathogenic species are *Y.pestis*, *Y.pseudotuberculosis*, *Y.ruckeri* and *Y.enterocolitica*, the latter one is an intestinal pathogen and the most prevalence *Yersinia* species among humans (Doyle and Beuchat 2007).

2.5.2 *Yersinia enterocolitica* infections in humans

Situation of Yersinia enterocolitica infections in humans

The incidence of *Y.enterocolitica* infections differ from report and region. In 2009, its incidence was 0.32 per 100,000 population or 150 cases from 17,468 laboratory-confirmed cases of infection among 10 US-states (CDC 2010), while the ECDC presented an overall notification rate of 2.88 per 100,000 population in 2007. This is 2 % less than the total number reported in 2006 (ECDC 2009). In Germany, the incidence of yersiniosis in 2008 was 5.3 per 100,000 population, which indicates a decrease compared to 2007 (6.1 per 100,000 population). The predominant serotype was O:3 (88%, n=3,359), followed by O:9 (7 %, n=277), O:5,27 (0,8 %, n=30) and O:8 (0,5 %, n=18) (Robert-Koch-Institut 2009).

Transmission of Yersioniosis

Y.enterocolitica causes sporadic cases in humans, which outbreaks are rarely reported. During 2000-2006 in southern Germany, the most important infection source was suggested to be contaminated pork and pork products (Bucher et al. 2008). Particularly ready-to-eat pork product was the probable source of a *Y.enterocolitica* O:9 outbreak in Norway (Grahek-Ogden et al. 2007). The highest isolation rate of *Y.enterocolitica* (all of them were biotype (BT4) O:3) was found in tonsils of slaughter pigs (67%), while no pathogenic strain was isolated from cattle, sheep, turkeys, and horses (Bucher et al. 2008). In Japan, 14 outbreaks of *Y.enterocolitica* were associated with salads. Isolation of serotype O8 from humans and rodents have been reported (Sakai et al. 2005).

Clinical manifestation

FoodNet reported the highest incidence rate among children aged <4 years. The percentage of hospitalized patients caused *Y.enterocolitica* by was highest among those aged ≥50 years (43.2%). The case fatality rate (CFR) was highest in persons aged ≥50 years (5.4%). In comparison with 1996-1998, the rate of infection in 2009 decreased for 53% (CDC 2010).

Symptoms in children include fever (50% higher than 39^{o}C), strong abdominal pain, diarrhea and vomiting (Sakai et al. 2005). Abdominal pain and diarrhea were also found in adult patients (Babic-Erceg et al. 2003).

2.5.3 *Y.enterocolitica* in animals

The intestinal carriage from pigs at slaughterhouses in Great Britain contained *Y.enterocolitica* in a proportion of 10.2% (Milnes et al. 2008) and 26.1% (McNally et al. 2004). The highest risk period of this pathogen carriage was found during December to May. Moreover, holding the animal overnight at the abattoirs was a risk factor of carriage (Milnes et al. 2009).

In Great Britain, *Y.enterocolitica* was isolated from intestines of cattle at slaughterhouses in a proportion of 4.5% (Milnes et al. 2008) and 6.3% (McNally et al. 2004). The carriage of *Y.enterocolitica* in cattle was strongly influenced by season. An increased risk was observed from December to May. Age was also shown as an influential factor with an increased risk in older cattle (Milnes et al. 2009). Other animals were identified to carry non-pathogenic *Y.enterocolitica* with a prevalence of 5% (dogs), 3% (cats) and 3% (rodents) using real-time PCR in southern Germany (Bucher et al. 2008).

In Great Britain, the most frequent biotype 1a was isolated predominantly from livestock (58%) and humans (53%). Pathogenic *Y.enterocolitica* isolated

from livestock was biotype BT3 (O5,27) (35% sheep, 22% pig and 4% cattle), which was not detected in any human isolates. The major pathogenic biotypes of strains isolated from humans were BT3 (O:9) 24%, BT4 (O:3) 19%, whereas 11% of pigs carried BT3 (O:9) strains. These data suggest that pigs may be a reservoir for human pathogenic *Y.enterocolitica* infections (McNally et al. 2004).

2.5.4 *Y.enterocolitica* along the lamb production chain

Y.enterocolitica serotype O:2,3 was the predominant serovar among sheep flocks in Australia. The isolation rate was influenced by season and age. *Y.enterocolitica* excretion occurred year-round, with the greatest prevalence being in summer and autumn. Moreover, sheep less than 1 year old were most commonly infected with *Y.enterocolitica* (Slee and Skilbeck 1992).

Y.enterocolitica has been rarely reported from investigations of lamb production. Fecal carriage of *Y.enterocolitica* by sheep at slaughterhouses in Great Britain was 10.7% (McNally et al. 2004). In another study from the same region, the organism was isolated from sheep intestines (8.0%) collected at slaughterhouse level (Milnes et al. 2008). The 6 month period from December to May was observed as a higher risk factor. Feeding during the lairage was also shown to be of risk (OR 2.00, 95%CI 1.03-3.85, P=0.048) (Milnes et al. 2009). In contrast, in a study from Greece, *Y.enterocolitica* was not found among sheep carcass samples (Kechagia et al. 2007).

3. Materials and Methods

Scope of study

This study was performed from July 2008 to August 2009. A total of 321 samples was collected from 9 sheep farms, supplying their animals to the same slaughterhouse. Samples were examined using qualitative methods (detection of thermophilic *Campylobacter, Listeria, E.coli, Salmonella* and *Yersinia*), whereas quantitative methods (Aerobic Plate Count and *Enterobacteriaceae* count) were applied only in the investigation of drinking water samples.

3.1 Materials

3.1.1 Sample collection

3.1.1.1 Sampling plan

In total, 14 visits were undertaken. The number of environmental samples and samples from the animals are included in Table 3.1. Samples from the first visit contained only fecal samples, while samples from the second one contained only lymphnodes. From the third to the fifth visits, samples were collected from the same farm. Starting with the sixth up to the fourteenth visits, samples were taken from 8 different farms.

Environmental samples included drinking water, the insulation from waterpipeline, feed, feed troughs, mineral stones, mineral powder, litter, roughage, roughage troughs and claw baths.

Samples from animals, including fleece, feces and lymphnodes, were also categorized as animal samples from the farm. Collection of the 3 latter ones took place at the slaughterhouse after transport and slaughter.

3.1.1.2 Sampling technique

Fleece

Fleece samples were collected during the bleeding procedure with sterile scissors and forceps and given in a plastic bag.

Fecal and lymphnode samples

After evisceration, fecal samples were collected directly from the large intestines, mesenteric lymphnodes were aseptically taken. Fecal samples were put in a plastic container, while lymphnode samples were given in plastic bags.

Claws

From the third excursion, two claw samples were collected using sterile cotton swabs. These samples were taken during lairage at the slaughterhouse.

Feed, roughage, mineral stones und powder

Concentrated feed and roughage were taken directly from the feed troughs and drinking water troughs at different sites. Mineral powder or swabs from mineral stones were also collected if available.

Drinking water

Drinking water samples were collected directly from water troughs or drinking automates. To collect drinking water samples, a sterile plastic bottle was used.

Feed troughs, drinking water troughs and insulation of waterpipeline

Gauze swabs were used to collect these samples at sites, which had contacted with animals. One insulation from waterpipeline was collected at the last excursion as it deemed not clean.

Litter

Litter, which had directly contacted with animals, was randomly collected from different sites.

3.1.1.3 Sample transportation

All samples were transported immediately to the laboratory at 0-4^{o}C within 2 hours, where they were recorded into the laboratory recording book. Samples were kept at 4^{o}C in a refrigerator and processed within 24 hours.

Table 3.1: Number of samples during July 2008 to August 2009

No. of excursion	collecting date	environmental samples										animal samples				total
		drinking water	waterpipeline insulation	feed	feed trough	mineral stone	mineral powder	litter	roughage	roughage trough	claw bath	claw	feces	lymphnode	fleece	
1	23/07/08												6			6
2	15/09/08													6		6
3	05/11/08	2						1	1			2	2	3	2	13
4	24/11/08	2		2	2			2	4				2		2	16
5	11/12/08	2		2	2			2	1				2	10	2	23
6	19/01/09	2				1		2	2				2	2	2	13
7	31/01/09			3		1		1	1				2	2	2	12
8	07/02/09	2		4		1		2	1		1		2	2	2	17
9	21/02/09	3		3		2	1	2	3	2			10	10	8	44
10	20/03/09	2		1	2	2		2	2				10	10	2	33
11	29/04/09	2		3	1	1		2	4	3			10	10	2	38
12	01/06/09	2		2	2			2	1	1			10	10	2	32
13	09/07/09	2		1	2	2		2	2	1			10	10	2	34
14	03/08/09	2	1	2	3			2	1	1			10	10	2	34
	total	23	1	23	14	10	1	22	23	8	1	2	78	85	30	321

3.2 Methods

3.2.1 Preparation of samples

Fleece

2 gram of fleece was given into 100 ml Buffered Peptone Water (BPW; Merck®) and homogenized with a stomacher for 60 seconds.

Fecal and lymphnode samples

A representative of 10 gram fecal materials was given into 90 ml BPW in a sterile stomacher bag. These samples were homogenized using a stomacher for 60 seconds.

Claws

Two cotton swabs taken from claws were put into 9 ml ONE-broth and were investigated only for the presence of *Listeria*.

Feed, roughage, mineral stones and mineral powder

10 gram of feed, 1 gram mineral powder and 5 gram roughage were added to 90, 100, 100 ml BPW respectively. These samples were then homogenized using a stomacher for 60 seconds.

Drinking water

10 ml of drinking water was added to 90 ml BPW, and thoroughly mixed.

Feed troughs, drinking water troughs and waterpipeline insulation

Gauze swabs from these samples were filled with 100 ml BPW and homogenized with a stomacher for 60 seconds.

Litter

5 gram litter was cut into small pieces and filled up with 100 ml BPW. Samples were homogenized using a stomacher for 60 seconds.

3.2.2 Qualitative investigation for zoonotic agents

3.2.2.1 Thermophilic *Campylobacter*

3.2.2.1.1 Identification of thermophilic *Campylobacter*

According to ISO 10272:2004, samples were diluted into 1:10. One ml of homogenized samples was selectively enriched in 9 ml Bolton broth, resuscitated at 37^{o}C for 4 h under aerobic conditions and incubated at 42^{o}C for 48 h under microaerobic conditions (10%CO_2, 5%O_2, and 85%N_2). These samples were inoculated on *Campylobacter* Blood free selective media (mCCDA) agar at 42^{o}C for 48 h under microaerobic conditions (Appendix 9.2.1.1).

3.2.2.1.2 Confirmation of thermophilic *Campylobacter*

Suspected *Campylobacter* colonies on mCCDA medium are grey or white in color. They were subcultured on Columbia blood agar and incubated at 42^{o}C for 48 h under microaerobic conditions (Appendix 9.2.1.1-2).

Biochemical tests (Table 3.2), including catalase, oxidase, motility test, carbohydrate decomposition profile, hippurate hydrolysis and susceptibility to nalidixic acid and cephalotine, were used to differentiate between genus and species (Appendix 9.2.1.2). Gram staining, oxidase test and catalase test were performed as described in Appendix 9.1.1-3. The culture from Columbia blood agar is not recommended to use for catalase test, so a culture from TSI (Triple Sugar Iron) agar was used for the catalase test.

Motility test

The "Hanging drop" technique was applied to test the motility of the strain. Colony was suspended in Brucella broth and a small drop of bacterial suspension was dropped on a coverslip. Petroleum gel was placed at each corner of the coverslip and a well slide with a central depression was inverted over the coverslip. The coverslip sticking to the

slide was inverted and the drop of bacterial culture was observed under x400 magnification with a microscope.

Triple sugar iron (TSI)

Triple sugar iron (TSI) was used for fermentation of glucose, lactose and sucrose and for the production of hydrogen sulfide (H_2S). The suspected colony was inoculated by stabbing into the butt the agar and by streaking on the slant, after that incubation took place at 42^{o}C 48 h under microaerobic conditions.

The interpretation of TSI took place under use of a standard. The slant reaction followed by the butt reaction, both of them was separated by a slash mark. The slant reaction was observed for the fermentation of lactose and/or sucrose, which is indicated by acidity (inverting the indication of phenolred from red to yellow). In addition, the butt reaction was observed for the fermentation of glucose, formation of H_2S and gas. Red color was identified as alkalinity (K) or a negative reaction, while the yellow color was identified as acidity (A) or a positive reaction.

- ***Glucose (K/A)***

  If only glucose is fermented, acid is produced in the butt. The butt turns yellow, while the slant will remain red.

- ***Lactose or sucrose (A/A)***

  If lactose and/or sucrose are fermented, a large amount of acid turns both butt and slant to yellow. Thus, this reaction indicates the ability of the culture to ferment either lactose or sucrose.

25^{o}C microaerobic growth

The suspected colony was inoculated in brucella broth and incubated at 25^{o}C for 48 h under microaerobic conditions. The turbidity of the broth was recorded.

Table 3.2: Biochemical tests for thermophilic *Campylobacter* species

test	*C. jejuni*	*C. coli*	*C. lari*	*C. upsaliensis*
catalase	+	+	+	-/(+)
oxidase	+	+		
motility	+	+		
25° C growth	-	-	-	-
glucose	-	-	-	-
sucrose	-	-	-	-
lactose	-	-	-	-
gas formation	-	-	-	-
H_2S	-	-	-	-

Source: (ISO10272:2004)

3.2.2.1.3 Identification of thermophilic *Campylobacter* species

Hippurate-hydrolysis

The suspected colony was inoculated in 0.4 ml 1.0% (w/v) sodium hippurate solution and incubated at 37^{o}C for 2 h. Then, 0.2 ml of a 3.5% (w/v) ninhydrin solution, freshly prepared, was added to cover the hippurate solution. Interpretation was done after incubation for 10 min at 37^{o}C incubation. A positive result is indicated by a dark violet or blue, whereas a negative result shows no color change or a light blue one.

Sensitivity testing for nalidixic acid and cephalotin

The suspected colony was suspended in Brucella broth in a concentration of 0.5 McFarland. This suspension was poured on the surface of Muller-Hinton blood agar and spread with a sterile spreader. Then 2 discs containing nalidixic acid and cephalothin were placed on this agar. All was incubated at 37^{o}C 24 h under microaerobic conditions. A clear zone indicates susceptibility, while resistance appears without any clear zone.

Table 3.3: Identification of *Campylobacter* species using biochemical tests

test	*C. jejuni*	*C. coli*	*C. lari*	*C. upsaliensis*
hippurate-hydrolysis	+	-	-	-
nalidixic acid(30 µg)	S	S	R	S
cephalotin (30 µg)	R	R	R	S

Source: (ISO10272:2004)

3.2.2.1.4 Identification of *Campylobacter* using Polymerase Chain Reaction (PCR)

DNA preparation

DNA was prepared by using approximately half a loopful of culture in 1 ml of distilled water. The procedure for DNA preparation was described in Appendix 9.3.1. These DNA were kept at -20° for PCR.

Selection of primers

Four pairs of primers were selected from published articles as described in Table 3.4. The primers "23SF and 23 SR" were used to detect *23S rRNA* for identification of the genus *Campylobacter*. The other primers were used to detect the genes *hipO* from *C. jejuni*, *glyA* from *C. coli* and *23S rRNA* from *C.lari*.

Table 3.4: Primers used for *Campylobacter* identification (PCR)

target gene	primer	Sequence (5'→ 3')	product size (bp)	reference
C. jejuni 23s rRNA	23SF 23SR	TATACCGGTAAGGAGTGCTGGAG ATCAATTAACCTTCGAGCACCG	650	(Wang et al. 2002)
C. jejuni hipO	CJF CJR	ACTTCTTTATTGCTTGCTGC GCCACAACAAGTAAAGAAGC	323	(Wang et al. 2002)
C.coli glyA	CCF CCR	GTAAAACCAAAGCTTATCGTG TCCAGCAATGTGTGCAATG	126	(Wang et al. 2002)
C.lari 23s rRNA	Therm1 lari	TATTCCAATACCAACATTAGT CTCTTAACGACTACGGCA	260	(Eyers et al. 1993)

PCR conditions

Three PCR reactions were applied to identify *Campylobacter*. The first one was a single PCR to identify a sequence from the 23s rRNA gene, in order to indicate the genus *Campylobacter*. The second one was a Duplex PCR, which differentiates *hipO* gene of *C.jejuni* and *glyA* gene of *C.coli*. The last single PCR was to indentify *23s rRNA gene* of *C.lari* (Table3.4).

The PCR procedures are described as follows:

- ***Identification of the genus Campylobacter***

The first single PCR used primer 23SF and 23SR as described in Table 3.4. PCR concentration and PCR condition were taken from Wang et al (2002). For 25 µl PCR reaction, the concentration of each reagent is as follows:

- 200 µM deoxynucleoside triphosphate
- 10× reaction buffer (500 mM Tris-HCl, 100 mM KCl, 50 mM $(NH_4)_2SO4$ and 20 mM $MgCl_2$)
- 0.5 µM of each 23SF and 23SR primer
- 0.5 unit of FastStart® Taq DNA polymerase (Roche Diagnostics, GmbH, Mannheim, Germany)
- 2.5 µl of whole-cell template DNA

DNA amplification was carried out in a thermocycler (Primus 96 plus, MWG®):

- Initial denaturation step at 95°C for 6 min.
- 30 cycles of
  - denaturation at 95°C for 0.5 min.
  - annealing at 61°C for 0.5 min.
  - extension at 72°C for 0.5 min.
- Final extension at 72°C for 7 min.

- ***Seperating between C.jejuni and C.coli***

The 2 primer pairs of *hipO* from *C.jejuni* and *glyA* from *C.coli* were used in the duplex PCR. These 2 PCRs were applied under the same conditions as described by Wang et al (2002) (Table 3.4). For 25 µl PCR reaction, the concentration of each reagent is described as follows:

- 200 µM deoxynucleoside triphosphate
- 10× reaction buffer (500 mM Tris-HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$ and 20 mM $MgCl_2$)
- 0.5 µM of each CJF and CJR primer for *C. jejuni* or
- 1 µM of each CCF and CCR primer for *C. coli*
- 0.5 unit of FastStart® Taq DNA polymerase (Roche Diagnostics, GmbH, Mannheim, Germany)
- 2.5 µl of whole-cell template DNA

DNA amplification was carried out in a thermocycler (Primus 96 plus, MWG®):

- Initial denaturation step at 95°C for 6 min.
- 30 cycles of amplification
  - denaturation at 95°C for 0.5 min.
  - annealing at 61°C for 0.5 min.
  - extension at 72°C for 0.5 min.
- Final extension at 72°C for 7 min.

- ***C.lari identification***

This single PCR for *C.lari* was taken from Eyers et al (1993) (Table 3.4). For 25 µl of the PCR reaction, the concentration of each reagent is described as follows:

- 200 µM deoxynucleoside triphosphate
- 10× reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, and 50 mM $[NH_4]_2SO_4$)
- 1.5 mM $MgCl_2$
- 0.25 µM of each Therm1 and Lari primer for C.lari
- 0.5 unit of AmpliTaq® Gold DNA Polymerase (Roche Molecular Systems, New Jersy, USA)
- 2.5 µl of whole-cell template DNA

DNA amplification was carried out in a thermocycler (Trio-thermobloack, Biometra®) as follows:

- Initial denaturation step at 95°C for 8 min.
- 45 cycles of amplification
    - denaturation at 94°C for 1 min.
    - annealing at 56°C for 1 min.
    - extension at 72°C for 1 min.
- Final extension at 72°C for 5 min.

Agarose gel electrophoresis

The 1.5% agarose gelelectrophoresis was performed in 1xTBE for 60 min. This gel was stained with 0.005% w/v Ethidium bromide and inspected under UV light. Results were recorded using Gel Documentation (DIAS-II, Serva®). The amplified products were assessed under simultaneous use of a DNA marker (pBR328, Roth®) at 650, 323 and 126 bp for genus *Campylobacter*, *C.jejuni* and *C.coli*, respectively. For *C.lari*, the amplified product was characterized at 260 bp by comparing with DNA markers (DNA-*Hind III* and Φx174 DNA-*Hae III*, Finnzymes®).

3.2.2.1.5 Molecular characterization using Pulsed Field Gel Electrophoresis (PFGE)

The PFGE procedure of Campynet (a Network project providing standardised molecular typing methods for the major foodborne pathogens *Campylobacter jejuni* and *Campylobacter coli*) was applied in this study (On et al. 2000).

a. Recovery of Campylobacter isolates

Of the 68 *Campylobacter* isolates, 64 isolates could be resuscitated after storage at -20^{o}C. The isolates were recovered in BHI broth at 42^{o}C for 48 h under microaerobic conditions. Then the inoculated broths were subcultured on Columbia blood agar and incubated at 42^{o}C under microaerobic conditions. After 48 h, pure cultures were harvested and used for the further process.

b. Preparing and embedding DNA

The bacterial mass was suspended in cold Pett-IV solution in order to adjust the concentration to 6-7 MacFarland using a spectrophotometer. Then 150 µl of this cell suspension was mixed with 350 µl 1% Agarose (Megabase, BioRad®). Cells in agarose suspension were filled in a plug mold (Biorad®) and these blocks were kept in a refrigerator for 10 min.

c. Bacterial cell lysis

After solidification of the suspension, this gel was transferred from the plug mold in an eppendorf tube. 1.5 ml ESP solution was added and incubated at 56^{o}C for 48 h. After 24 h of incubation, this solution was replaced with freshly prepared ESP solution.

d. Washing steps and chromosomal DNA restriction

After 2 days incubation of lysis, the ESP solution was removed and gel blocks were washed for 6 times, by adding 1300 µl TE buffer (pH 8) and shaking for 20 min each time. From the block, 1 mm gel were cut and

taken into a new eppendorf tube. Then the restriction enzyme "Kpn-I (Roche®)" was added and all was incubated at 37°C 24 h.

e. Gel electrophoresis

The DNA fragments were separated in a 1.4 % agarose gel (Ultra pure DNA grade agarose, BioRad®) in 0.5× TBE buffer (45 mmol of Tris, 45 mmol of boric acid, 1 mmol of EDTA) at 200 Volts. Seperation took place with a ramped pulse from 4 to 20 s for 20 h. In all gels, a PFGE marker (Pulse marker 50-1,000 kb, Sigma®) was used as a standard molecular weight marker. Restriction patterns were inspected visually for identification or relation.

f. Interpretation of restricted DNA

The DNA restriction patterns were interpreted based on the criteria from Tenover (1995). Patterns were identical, if the bands were in the same sizes by means of position and strength the corresponding bands (Appendix 9.3.2) (Tenover et al. 1995).

In case of 1-3 fragments difference (The sum of the size of 2 smaller fragments should be approximate the size of the larger fragments), the interpretation was defined as "subtype". Such subtypes indicate closely related PFGE-types and subtypes (Appendix 9.3.2) (Tenover et al. 1995).

3.2.2.2 *Listeria* spp.

3.2.2.2.1 Identification of *Listeria* spp.

ISO 11290:1995 was applied for identification of *Listeria* spp. One ml of the homogenized sample was given into 9 ml ONE broth (OXOID Novel Enrichment (ONE) Broth-*Listeria*). In case of growth, black color was observed after 24 or 48 h. If black color occurred within 24 h, these samples were subcultured on Oxoid Chromogen *Listeria* selective agar

(OCLA). After 48 h, the rest of samples was inoculated on OCLA agar and incubated at 37°C for 48 h (Appendix 9.2.2.1- 2).

3.2.2.2.2 Confirmation of *Listeria* spp.

A presumptive *Listeria* colony appears as a blue colony on OCLA agar. Such a colony was subcultured on Columbia blood agar and standard I agar (Merck®), then both of them were incubated at 37°C 48 h (Appendix 9.2.2.1-2).

Gram staining, oxidase test and catalase test were performed as described in Appendix 9.1.1-3. Biochemical tests for genus identification and species differentiation were applied and recorded (Appendix 9.2.2.3-4).

Hydrogensulfid formation

Kligler was used to test for H_2S formation. The suspected colony was inoculated by stabbing and streaking as described for TSI for *Campylobacter* confirmation (3.2.2.1.2). However, the incubation was performed at 30°C 24 hr under aerobic conditions. H_2S formation is indicated by a black color formation in the bottom of the tube.

Voges-Proskauer reaction

The colony was inoculated into the Voges-Proskauer broth and mixed by vortex and the mediumwas incubated at 37°C for 48 h. 3 ml Barrit reagent (alpha-naphtol solution) was added and mixed by vortex. Then 1 ml 40% Potassium hydroxide solution was added and mixed by vortex. The result was analyzed within 15 min, pale pink to strong red indicates a positive reaction. In case of a negative reaction presents, a colourless, yellow or brown yellow appears.

Methyl red test

The colony was inoculated into Voges-Proskauer broth and mixed by vortex. After incubation at 37°C for 48 h, a few drops of methylred solution were added. A red color is positive, while a negative result shows a yellow color.

Urease test

The suspected colony was stabbed into urea agar and streaked on the slant agar, the medium was incubated at 37^{o}C for 48 h. A positive result shows a pink color, while a yellow color indicates a negative result.

Oxidative–Fermentative test

A suspected colony was stabbed into the bottom of 2 tubes with semisolid media (glucose as the substance for destruction). One tube was covered with sterile liquid paraffin, whereas the other one was not. Both were incubated at 37^{o}C for 48 h. The paraffin-covered tube represents the fermentative reaction, while the uncovered tube shows the oxidative reaction. In a case of doubt, they were incubated for several days. A positive result shows a yellow color, while a green color presents a negative result.

Esculin test

The suspected culture was inoculated into the esculin broth and mixed by vortex. After incubation at 37^{o}C for 48 h, a few drops of 10% iron citrate solution were added. In case of a positive result, a black color occurrs.

Indol test

The suspected colony was inoculated into the broth and mixed by vortex. After incubation at 37^{o}C for 48 h, the broth was covered with 0.5 ml of Kovac's reagent. In case of a positive result, a red color appears on the cover part after little shaking.

Motility test

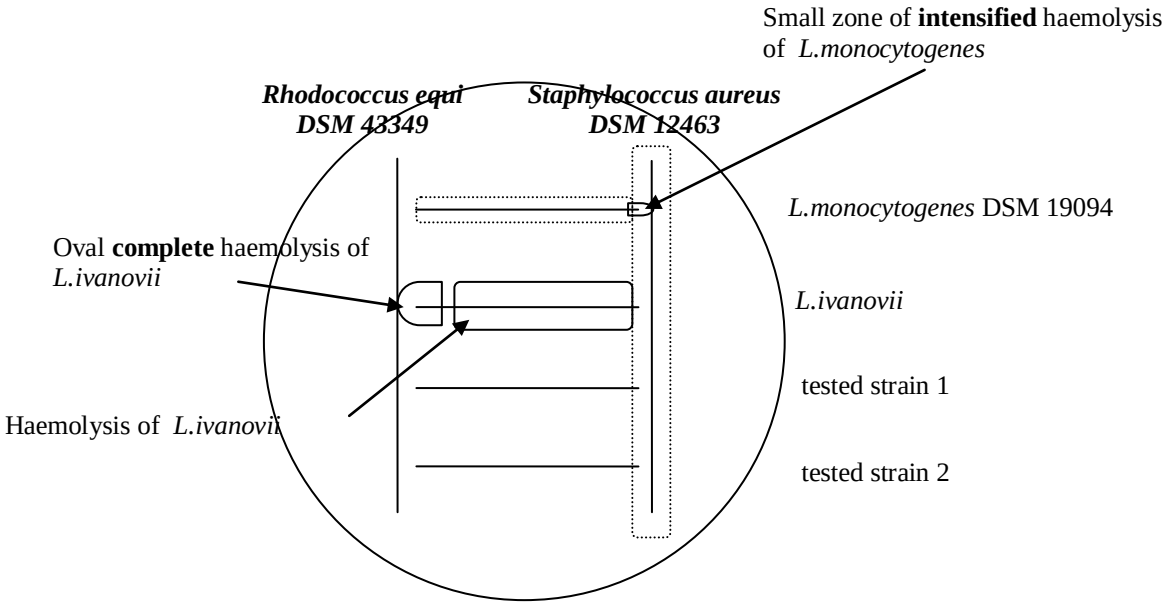
The suspected colony was inoculated with needles in a semisolid medium about 0.5 – 1 cm, and incubated at 25°C 2-5 days. The motility was deduced from the cloudy zone around the inoculation stab. This observation can also be done after a longer period.

3.2.2.2.3 Identification of *Listeria* species by biochemical tests

CAMP test

5% sheep blood agar No.2 was used to perform the CAMP test. Cultures of *Staphylococcus aureus* und *Rhodococcus equi* were streaked right and left as vertical lines on the agar plate. Then the reference strains of *L.monocytogenes* und *L.ivanovii* were streaked as horizontal lines, with 2-3 mm distance between horizontal and vertical lines. Test strains were inoculated also as a horizontal line as shown in Figure 3.1. The agar was incubated 24 hr at 37^{o}C. The type of haemolysis between the test strain with *Staphylococcus aureus* and *Rhodococcus equi* was recorded (See Figure 3.1).

Figure 3.1: The CAMP test for *Listeria* species differentiation



Carbohydrate fermentation

Utilization of carbohydrates, including rhamnose, d-xylose, arabinose, and mannitol, was used to confirm the species. The suspected colony was inoculated in the respective broth and mixed by vortex. These broths were incubated at 37^{o}C for 48 h. A positive result shows a yellow color, while a negative result is presented by a violet color. Interpretation of results is done as described in Table 3.5.

Nitrate test

The colony was inoculated in nitrate broth and mixed by vortex. After incubation at 37^{o}C 48 h, a few drops of Griess-Ilosvay solution were added. A positive result showed pink color at that moment, this color may disappear. In case of no color change, a second reaction must be applied by adding small amount zinc. No color change indicates a positive result, while the appearance of pink color indicates a negative result.

Table 3.5: Identification of *Listeria* species by biochemical tests

substance	*L.mnocytogenes*	*L.innocua*	*L.seeligeri*	*L.welshimeri*	*L.ivanovii*	*L.grayi*	*L.murrayi*	*L.denitrificans*	positive	negative
CAMP										
* St.aureus	+	-	+	-	-	-	-	-		
* Rh.equi	-	-	-	-	+	-	-	-		
rhamnose	+	D	-	D	-	-	D	-	yellow	violet
d-xylose	-	-	+	+	+	-	-	+	yellow	violet
arabinose	-	-			-	-	-	+	yellow	violet
mannitol	-	-	-	-	-	+	+	-	yellow	violet
nitrate	-	-	-	-	-	-	+	+		
hemolysis*	+	-	+	-	+	-	-	-		
VP*	+	+	+	+	+	+	+	-		

(Source: (ISO11290:1995)

* Hemolysis was observed from columbia blood plate. Voges-Proskauer reaction was performed for Genus confirmation

3.2.2.3 *Escherichia coli*

3.2.2.3.1 Isolation of *Escherichia coli*

One ml of homogenized samples was enriched in 10 ml of selective Brilliant Green 2 % Bile Broth (Brila) and incubated at 43^{o}C for 48 h. Enriched broths were inoculated on *E.coli* Direct agar (ECDC) agar and incubated at 43^{o}C for 24 h (Appendix 9.2.3.1-2).

3.2.2.3.2 Confirmation of *Escherichia coli*

Colonies from ECD agar were used for detecting *E.coli* by appearance of fluorescence under UV light (366 nm) and by indol test (Appendix 9.2.3.2).

Fluorescence test

The ECD agar plate was inspected directly under 366 nm UV light. In case of a positive result, fluorescence was observed. In case of negative, the respective plate was left at room temperature and was re-inspected after 48 h.

Indol test

If the fluorescence test was positive, the indol test was applied. A few drops of Kovac's reagent were dropped directly on ECD agar plate. A positive result appears as a red color on the colony, while a negative result has no change.

3.2.2.3.3 Identification of *E.coli* using Polymerase Chain Reaction (PCR)

DNA preparation

Approximately half a loopful of a culture was transferred into 1 ml of distilled water for DNA preparation. The whole cell procedure was

applied for DNA preparation as described in Appendix 9.3.1. DNA were kept at -20° for PCR.

PCR conditions

The primers EC1 and EC2 used are described in Table 3.6. PCR procedures were followed Osek et al. (2000). For 25 µl PCR reaction, the concentration of each reagent is described as follows:

- 200 µM deoxynucleoside triphosphate
- 10× reaction buffer (500 mM Tris-HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$ and 20 mM $MgCl_2$)
- 1 µM of each primer
- 0.5 U of FastStart Taq DNA polymerase (Roche Diagnostics, GmbH, Mannheim, Germany)
- 2.5 µl of whole-cell template DNA

DNA amplification was carried out in a Perkin-Elmer thermocycler as follows:

- an initial denaturation step at 94°C for 5 min.
- 35 cycles of amplification
    - denaturation at 94°C for 1 min.
    - annealing at 56°C for 1 min.
    - extension at 72°C for 1 min.
- a final extension at 72°C for 5 min.

Table 3.6: Primers used for *E.coli* identification (PCR)

target gene	primer	sequence (5'→ 3')	product size (bp)	reference
uspA	EC1	CCGATACGCTGCCAATCAGT	884	(Osek et al. 2000)
	EC2	ACGCAGACCGTAAGGGCCAGAT		

Agarose gel electrophoresis

The amplified products were expected to appear at 884 bp using DNA marker (pBR328, Roth®). The 2.0% agarose gelelectrophoresis was performed in 1xTBE for 60 min. This gel was stained with 0.005% w/v Ethidium bromide and inspected under UV light, the result was recorded using Gel Documentation (DIAS-II, Serva®).

3.2.2.4 *Salmonella* spp.

3.2.2.4.1 Identification of *Salmonella* spp.

In order to resuscitate *Salmonella*, each sample was given in Buffered Peptone Water (BPW) as described above, and incubated at 37°C for 18-20 hr. According to ISO 6579:2002, Muller Kauffman Tetrathionat Novobiocin broth (MKTTn) and Rappaport and Vassiliadis broth (RV) were used as selective enrichment for *Salmonella* (ISO6579:2002). 1 ml and 0.1 ml of these BPW-enrichment samples were inoculated into MKTTn and RV, respectively. MKTTn was incubated at 37°C, while RV was incubated at 42°C. After 24 and 48 h, both of them were inoculated on Rambach® (Merck) and Brilliant-green Phenol-red Lactose Sucrose Agar (BPLS), both of them were incubated at 37°C for 24 h. Suspected colonies show red appearance on Rambach® agar and pink color on BPLS. Results were recorded from Rambach® and BPLS after 24 and 48 h. Suspected colonies are subcultured onto Standard-I agar (Merck®) and incubated at 37°C for 24 h (Appendix 9.2.4.1-2).

3.2.2.4.2 Confirmation of *Salmonella* spp.

The Kauffman-White scheme was applied to identify *Salmonella* spp. and the serovar of the isolates with commercial antiserum (Sifin®). The suspected colony from both selective medium is to be subcultured on Standard I agar (Merck®) and incubated at 37°C 24 h. Nutrient agar (Standard I agar; Merck®) is necessary for this *Salmonella* serological test,

because culture from a selective medium can affect the antigen structure of the isolates.

A small amount of bacterial mass is transferred to a slide. Mixing well with a drop of test reagent (about 25 µl), results in a homogenous, slightly milky suspension. Prior to the serological test, the strains were checked for autoagglutination with physiological saline solution as a negative control.

Under the rotating slide, the reaction can be observed with the naked eye by holding the slide in front of the light source against a black background. The result should be observed under dark or black background. Agglutination should be visible within 20 rotations in a positive case, the negative remains as a milky suspension. The result cannot be interpreted if the culture has a positive result with saline solution (autoagglutination).

3.2.2.5 *Yersinia enterocolitica*

3.2.2.5.1 Identification of *Yersinia enterocolitica*

ISO10273:2003 was applied to identify pathogenic *Yersinia enterocolitica*, Ossmer broth and Peptone Sorbitol and Bile salt (PSB) broth were used as selective enrichment. 1 ml of homogenized samples was transferred to 9 ml Ossmer broth, whereas 0.5 ml samples were inoculated into 9.5 ml PSB broth. Ossmer and PSB broth were incubated at 25^{o}C for 48 h and 5 days, respectively, aliquots from both of them were streaked on Cefsulodin Irgasan Novobiocin (CIN) agar. In addition, 0.5 ml of PSB broth was transferred into 4.5 ml KOH. After 20 seconds, material from there was subcultured on CIN agar. All of them were incubated at 30^{o}C for 24 h. *Yersinia* suspected colonies are small (≤ 1 mm) and smooth with a red center and translucent rim. When examined under the obliquely transmitted light, they are non-iridescent and finely granular (Appendix 9.2.5.1). Suspected colonies were subcultured on Standard-I agar (Merck®) and the results were recorded (Appendix 9.2.5.2).

3.2.3.5.2 Confirmation of *Yersinia enterocolitica*

For biochemical testing, suspected colonies from CIN agar must be subcultured on Standard I agar (Merck®) and incubated at 30°C 24 h. Gram staining and oxidase test were performed as described in Appendix 8.1. Biochemical tests, including *Yersinia enterocolitica* confirmatory tests (Table 3.7) and pathogenicity tests (Table 3.8) were applied and recorded (Appendix 9.2.5.3).

Table 3.7: Biochemical tests for identification of *Yersinia enterocolitica*

test	*Yersinia enterocoloitca*	positive	negative
oxidase	negative	blue	no color
H_2S formation	negative	black	No
lysin decarboxylase	negative	yellow	violet
orithin decarboxylase	positive	yellow	violet
urease	positive	pink	yellow
tryptophandesaminase	negative	brown	yellow
glucose	positive	yellow	red
lactose	negative	yellow	red
gas formation	negative	bubble	no
saccharose	positive	yellow	red
rhamnose	negative	yellow	red
trehalose	positive	yellow	red
citrat	negative	blue	green
tween-esterase	positive	precipitate	no

(Source: (ISO10273:2003)

Hydrogensulfid formation

Kligler is used to test for H_2S formation. The colony is inoculated by stabbing in the agar butt and by streaking on the slant agar. The medium was then incubated at 30°C 24 h. H_2S appears as black color formation on the bottom of the tube.

Lysin decarboxylase and Ornithin decarboxylase test

A suspected colony is to be inoculated in each broth and mixed by vortex, broths were incubated at 30^{o}C 24 h. The interpretation of the result is shown in Table 3.7.

Urease test

The suspected colony was inoculated in the urea broth, mixed by vortex and incubated at 30^{o}C 24 h. A positive result shows pink color, while in case of a negative result no change occurrs.

Tryptophandesaminase

Tryptophandesaminase was examined in the same broth as used for urease. A suspected colony was inoculated in urea broth, mixed by vortex and incubated at 30^{o}C 24 h. To read the urea result, 3 drops of 7.5% iron (III) chloride solution were added. Brown color indicated positive result.

Kligler's Iron Agar

Kligler's Iron Agar (KIA) is principally the same as TSI. In TSI, the primary difference is the addition of sucrose, the third carbohydrate. KIA is used to determine if bacteria can ferment glucose and/or lactose and if they can produce hydrogen sulfide (H_2S) or any other gas. The suspected colony was inoculated by stabbing in the agar butt and by streaking on the slant agar, after that incubated at 30^{o}C 24 h. Interpretation of KIA was already described by TSI in *Campylobacter* confirmation (3.2.2.1.2).

Carbohydrate utilization

The carbohydrate utilization, including saccharose, rhamnose and trehalose, was used to confirm the species. Confirmation of suspected colony was inoculated in the respective broth and mixed by vortex. Broths were incubated at 30^{o}C for 24 h. Red color indicates a positive, while yellow color indicates a negative result.

Citrate test

The suspected colony was stabbed into Simmons-citrate agar butt and streaked on the slant agar (incubation at 30^{o}C 24 h). A positive result indicates by blue color, no change will be observed in case of a negative result.

Tween-Esterase Test

Suspected colony was streaked on the slope phase of Tween-Esterase medium and incubated at 25^{o}C for 5 days. A positive reaction appears as an opaque zone of precipitate due to calcium oleate microcrystals. No change appears in case of a negative reaction.

3.2.3.5.3 Confirmation of pathogenicity of *Yersinia enterocolitica*

Pathogenic *Yersinia enterocoloitca* strains were identified using utilization of esculin, presence of pyrazinamidase and calcium dependence at 37^{o}C as shown in Table 3.8.

Table 3.8: Biochemical tests for pathogenicity of *Y.enterocolitica* strains

test	*Yersinia enterocoloitca* DSM 4780	positive	negative
esculin	-/+	black	No color change
pyrazinamidase	-	red	No color change
calcium dependence at 37^{o}C	+		

(Source: (ISO10273:2003)

Esculin

The colony was stabbed into the esculin agar butt and streaked on the slant agar, the reaction was incubated at 30^{o}C 24 h. Black color around the colony indicates a positive result.

Pyrazinamidase

The colony was stabbed into the pyrazinamidase agar butt and streaked on the slant agar. After incubation at 30°C for 24 h, 1 ml of 1% ammonium iron (III) sulphate solution was added. Red color develops on the slant agar in case of positive result.

Calcium dependence at 37°C

In order to provide a suspension of 10^3 bacteria per ml, a small amount of the suspected colony was suspended in sodium chloride solution. 0.1 ml of this suspension was given on 2 plates of casein-soya agar and 2 plates of casein-soya agar with magnesium and oxalate. This suspension was spread over the complete surface of each plate as soon as possible with a sterile spreader. One of each medium was incubated at 25°C for 48 h and 37°C for 48 h respectively.

The positive reaction is regarded as follows;

- if at 25°C the colonies are of uniform size,
- if at 37°C, in the presence of magnesium and oxalate, >20% of colonies are smaller (0.1 ml in diameter). The remainder are 0.5 to 1.0 mm in diameter.

These inhibited colonies are calcium dependent and are presumed to be pathogenic.

3.2.3 Quantification of bacteria

3.2.3.1 Preparation of dilutions

A total of $10^{-1} – 10^{-6}$ dilution steps was prepared, water samples were used as 10^{0} dilution. To prepare a decimal dilution, 1 ml of the initial dilution was given into 9 ml NaCl peptone solution as a diluent and mixed by vortex. Then decimal dilutions were performed by transferring 1 ml of the previous dilution to 9 ml NaCl peptone solution and mixed again by vortex. Separate sterile pipettes were used in each diluting step.

3.2.3.2 Surface plating technique

a. Total bacteria count

Dilutions from 10^{-1}-10^{-6} were used for the Aerobic Plate Count (APC). Beginning with the dilution (10^{-1}), 0.1 ml of a diluted sample was pipetted on duplicate Standard I (Merck®) agar plate. This suspension was spread over the complete surface of each plate as soon as possible with a sterile spreader. Separate sterile pipettes were used in each dilution step and separate sterile spreaders were used in each plate. Plates were left on the bench for approximately 10 minutes to allow absorption of the inoculum into the agar. After 30°C 48 hr incubation, plates were observed and counted for the number of colonies.

b. Enterobacteriaceae count

Dilutions from 10^{-1}-10^{-4} were used for Enterobacteriaceae count. Beginning with the first dilution (10^{-1}), 0.1 ml of the diluted sample was pipetted on duplicate Violet Red Bile Dextrose agar (VRBD). This suspension was spread over the complete surface of each plate as soon as possible with a sterile spreader. Separate sterile pipettes were used for

each dilution step and separate sterile spreaders were used for each plate. Plates were left on the bench for approximately 10 minutes to allow absorption of the inoculum into the agar. After 30^{o}C 48 hr under anaerobic conditions, plates were observed and counted for the number of colonies.

To confirm a colony forming unit as Enterobacteriaceae, suspected colonies were subcultured onto a segment of Standard I agar plate and incubated at 30^{o}C 24 hr. Each colony was tested for oxidase (Appendix 8.1.2). Only colonies with a negative result for oxidase were counted as Enterobacteriaceae.

3.2.3.3 Recalculation

The number of colony forming units was calculated as follows:

$$N = \frac{\sum C}{[\,(1 \times n_1) + (0.1 \times n_2)\,]} \times (d) \times 10 \times \frac{100}{10}$$

When N = Number of colonies per ml

$\sum C$ = Sum of all colonies on all plates counted

n_1 = Number of plates in first dilution counted

n_2 = Number of plates in second dilution counted

d = Dilution from which the first counts were obtained

*Detection limit of this method was 10^2cfu/ml. In case of results lower than detection limit, 10^1cfu/ml was used to calculate.

4. Results

4.1 Qualitative approach for zoonotic agents

From July 2008 to August 2009, a total of 321 samples was collected. Of these, 195 samples were samples of animal origin, while 126 were environmental samples (Table 4.1). Nine sheep farms were included in this study and all animals from these sites were transferred and slaughtered in the same abattoir.

Table 4.1: Number of collected samples from July 2008 to August 2009

sample		amount
animal	fleece	30
	fecal sample	78
	lymphnode	85
	claw	2
	total	195
environmental	claw bath	1
	feed	23
	feed trough	14
	roughage	23
	roughage trough	8
	mineral stone	10
	Mineral powder	1
	drinking water	23
	waterpipeline insulation	1
	litter	22
	total	126
	grand total	321

4.1.1 Thermophilic *Campylobacter*

4.1.1.1 *Campylobacter* identification based on conventional diagnosis

4.1.1.1.1 Identification of the genus *Campylobacter*

Of 321 samples, 68 isolates from 65 samples were identified as *Campylobacter* spp. using conventional microbiological techniques. The overall isolation rate was 20.4% (65/319). The prevalence found in animal samples (27.5%, 53/193) was

higher than the prevalence in environmental samples (9.5%, 12/126). *Campylobacter* was most frequently identified in fecal samples (43.6%, 34/78), followed by litter (40.9%, 9/22), fleece (20.0%, 6/30) and lymphnodes (15.3%, 13/85). Drinking water and feed were found *Campylobacter* positive in a lower rate (8.7% (2/23) and 4.3% (1/23), respectively). However, *Campylobacter* was not identified in other environmental samples, e.g. claw bath, feed troughs, roughage, roughage troughs, mineral stones, mineral powder, and waterpiepline insulation (Table 4.2).

Table 4.2: *Campylobacter* positive number of samples

sample		N	number of positive samples (%)	*Campylobacter* isolates
animal	fleece	30	6 (20)	7
	fecal sample	78	34 (43.6)	36
	lymphnode	85	13 (15.3)	13
	total	193	53 (27.5)	56
environmental	claw bath	1	0	0
	feed	23	1 (4.3)	1
	feed trough	14	0	0
	roughage	23	0	0
	roughage trough	8	0	0
	mineral stone	10	0	0
	mineral powder	1	0	0
	drinking water	23	2 (8.7)	2
	waterpipline insulation	1	0	0
	litter	22	9 (40.9)	9
	total	126	12 (9.5)	12
	grand total	319	65 (20.4)	68

4.1.1.1.2 *Campylobacter* species differentiation using biochemical tests

A total of 68 *Campylobacter* isolates had been stored at -20^{o}C before further investigation. Of these, 66 isolates were recovered and differentiated for *Campylobacter* species using biochemical tests. *C.jejuni* was predominantly found in all samples (66.7%, 44/66), while the total amount of *C.coli* was 30.3% (20/66). *C.lari* was isolated only in 2 fecal samples (3.0%, 2/66) (Table 4.3).

Among animal samples, *C.jejuni* was the predominant species (64.8%, 35/54), which was the case also in other samples, including fleece (57.1%, 4/7), fecal samples (58.8%, 20/34) and lymphnodes (84.6%, 11/13). *C.coli* was obtained in a lower rate in each sample, in particular fleece 42.9% (3/7), fecal samples 35.3% (12/34), lymphnodes 15.4% (2/13). In addition, *C.lari* was isolated only in 2 fecal samples (Table 4.3).

C.jejuni was also predominant in environmental samples (75%, 9/12). This species was identified in litter (66.7%, 6/9), whereas 1 isolate from feed and 2 isolates from drinking water were *C.jejuni* (100%). *C.coli* was isolated only in 3 isolates from litter 33.33% (3/9), whereas *C.lari* was not found in environmental samples at all (Table 4.3).

Table 4.3: *Campylobacter* species in different types of samples using biochemical tests

sample		*Campylobacter* isolates	number of species isolates using conventional methods (%)		
			C.jejuni	*C.coli*	*C.lari*
animal	fleece	7	4 (57.1)	3 (42.9)	-
	fecal sample	34*	20 (58.8)	12 (35.3)	2 (5.9)
	lymphnode	13	11 (84.6)	2 (15.4)	
total		54	35 (64.8)	17 (31.5)	2 (3.7)
environmental	claw bath				
	feed	1	1 (100)	-	-
	feed trough				
	roughage				
	roughage trough				
	mineral stone				
	mineral powder				
	drinking water	2	2 (100)	-	-
	waterpipeline insulation				
	litter	9	6 (66.7)	3 (33.3)	-
total		12	9 (75)	3 (25)	-
grand total		66	44 (66.7)	20 (30.3)	2 (3.0)

* 2 isolates could not be cultivated to recover after storage at -20°C.

4.1.1.2 *Campylobacter* identification using Polymerase Chain Reaction

4.1.1.2.1 Identification of the genus *Campylobacter* by PCR

In order to compare the identification of the genus using conventional methods with PCR, a PCR was applied to all isolates. A total 63 of 68 isolates (92.6%) were positive using PCR method as described in Table 4.4.

Table 4.4: Comparison between the genus-identification using conventional microbiological methods with Polymerase Chain Reaction

sample		conventional microbiological methods	Polymerase Chain Reaction
animal	fleece	7	6
	fecal sample	36	35
	lymphnode	13	11
	total	56	42
environmental	claw bath		
	feed	1	1
	feed trough		
	roughage		
	roughage trough		
	mineral stone		
	mineral powder		
	drinking water	2	2
	drinking water insulation		
	litter	9	8
	total	12	11
	grand total	68	63

4.1.1.2.2 *Campylobacter* species differentiation by PCR

Regarding all samples, the majority of *Campylobacter* species was *C.jejuni* 35.3% (24/68), followed by *C.lari* 30.9% (21/68) and *C.coli* 26.5% (18/68).

Among animal samples, *C.lari* was the predominant species with 35.7% (20/56). The isolation rate of *C.coli* was 30.4% (17/56), while *C.jejuni* was the lowest fraction 26.7 (15/56). Furthermore, 1 isolate from a fecal sample was found to be a mixed culture from *C.jejuni* and *C.lari*, while another isolate from a fecal sample was identified as other *Campylobacter* species. 1 isolate from the fleece and 1 isolate from lymphnode were negative with all primers.

C.jejuni was the majority species found among environmental samples 75% (9/12). Similar to *C.lari*, *C.coli* was found in only 1 isolates from litter (Table 4.5).

Table 4.5: The differentiation of *Campylobacter* using species-specific PCR

sample		*Campylobacter* isolates	number of isolates in each species using Polymerase Chain reaction(%)					
			C.jejuni	*C.coli*	*C.lari*	Mixed *C.jejuni*-*C.lari*	Other spp.	negative
animal	fleece	7	2 (28.6)		4 (57.1)			1 (14.3)
	fecal sample	36	3 (8.3)	17 (47.2)	14 (38.9)	1 (2.8)	1 (2.8)	
	lymphnode	13	10 (76.9)		2 (15.4)			1 (7.7)
total		56	15 (26.7)	17 (30.4)	20 (35.7)	1 (1.8)	1 (1.8)	2 (3.6)
environmental	claw bath							
	feed	1	1 (100)					
	feed trough							
	roughage							
	roughage trough							
	mineral stone							
	mineral powder							
	drinking water	2	2 (100)					
	waterpipeline insulation							
	litter	9	6 (66.7)	1 (11.1)	1 (11.1)	1 (11.1)		
total		12	9 (75)	1 (8.3)	1 (8.3)	1 (8.3)		
grand total		68	24 (35.3)	18 (26.5)	21 (30.9)	2 (2.9)	1 (1.5)	2 (2.9)

4.1.1.3 *Campylobacter* strain Characterization using Pulsed Field Gel Electrophoresis (PFGE)

A total of 68 *Campylobacter* isolates had been stored at -20^{o}C. Of these, 62 isolates were further characterized using Pulsed Field Gel Electrophoresis with the enzyme *Kpn*-I. No restriction site was found in 5 isolates, while 4 isolates yielded many bands, which could not be interpreted. A total of 14 PFGE-types were characterized from 53 isolates (Appendix 9.3.2). The subtypes were found in PFGE-genotypes K-5 and K-7 and indicated as K-5.1 and K-7.1 (described in 3.2.2.1.5).

Associations between animal and environmental samples were observed only in some cases. The PFGE-type found in animal samples was also found in the environmental samples. The PFGE-type K-5 from the 7th excursion was obtained from fecal samples, feed and litter. Similarly to the PFGE-type K-6 from the 9th excursion, the PFGE-type K-1 from the 12th excursion was identified in both animal and environmental samples (Table 4.6).

In addition, horizontal relation between excursion sites was found in some PFGE-types. The PFGE-type K-2 was found in the 1st and the14th excursion. Similarly, type K-6 was found in 6th, 7th and 9th excursion (Table 4.6).

Moreover, associations between PFGE-types were also observed in the type K-7.1 in the 2nd excursion and type K7 in the 4th excursion. The type-K-5 in 7th excursion was similar to the type-K-5.1 in the 5th and the 8th excursion (Table 4.6).

Table 4.6: *Campylobacter* isolates characterized by PFGE typing and their occurrence during different excursions

sample		Excursion											
		1	2	4	5	6	7	8	9	10	12	13	14
animal	Fleece			K-5.1(3)					K-10(1)				
	fecal sample	K-2 (1) K-8 (1) K-14(1)		K-7 (1)	K-5.1(1)	K-6 (1)	K-5(1) K-6 (1)	K-11(1)	K-6(8)	K-9(1) K-12(1)	K-1(5)	K-3(1)	
	lymphnode		K-7.1(1)				K-6 (2)		K-6(3)		K-1(2) K-13(1)	K-3(1)	K-2(1)
environmental	feed						K-5(1)						
	drinking water										K-1(2)		
	litter						K-5(1)	K-5.1(1)	K-6(2)		K-1(2)		

*The PFGE-types K1-K14 were shown in appendix 9.3.2
The numbers of isolates found in each PFGE-type in brackets.

4.1.2 *Listeria* spp.

4.1.2.1 *Listeria* positive samples

Of the 321 samples, 51 isolates from 46 samples were identified as members of the genus *Listeria* (14.33%). The isolation rate was higher in environmental samples 27% (34/126), whereas the percentage of animal samples was 6.2% (12/195). Although mineral powder and pipelinewater insulation were collected only once, both of them harbored *Listeria* (100%, 1/1).

Among other environmental samples, *Listeria* was predominantly found in litter (59.1%, 13/22), followed by mineral stones (30%, 3/10), roughage (26.1%, 6/23), roughage troughs (25%, 2/8), drinking water (17.39%, 4/23), feeding troughs (14.3%, 2/14) and feed samples (8.7%, 2/23). *Listeria* was not identified in the claw bath sample. From animal samples, claw swabs were most frequently found to be positive with *Listeria* (50%, 1/2), while the occurence in other samples was 20% (6/30) in fleece, 3.5% (3/85) in lymphnodes and 2.6% (2/78) in fecal samples (Table 4.7).

Table 4.7: *Listeria* positive number of samples

samples	sample	N	number of positive samples (%)	*Listeria* isolates
animal	fleece	30	6 (20.0)	7
	fecal sample	78	2 (2.6)	2
	lymphnode	85	3 (3.5)	3
	claw	2	1 (50.0)	1
	total	195	12 (6.2)	13
Environmental	claw bath	1	0	0
	feed	23	2 (8.7)	3
	feed trough	14	2 (14.3)	2
	roughage	23	6 (26.1)	7
	roughage trough	8	2 (25)	3
	mineral stone	10	3 (30)	3
	mineral powder	1	1 (100)	2
	drinking water	23	4 (17.4)	4
	waterpipline insulation	1	1 (100)	1
	litter	22	13 (59.1)	13
	total	126	34 (27)	38
	grand total	321	46 (14.3)	51

4.1.2.2 Species differentiation of *Listeria* using biochemical tests

The majority of the *Listeria* isolates was identified as *L.monocytogenes* 43.1% (22/51), while *L.innocua*, *L.denitrificans*, and *L.ivanovii* was found in a percentage of 23.5% (12/51), 13.7% (7/51) and 9.8% (5/51), respectively. In addition, 5 *Listeria* isolates did not fit into the species diagnostic scheme and were identified as other *Listeria* spp. (Table 4.8).

L.monocytogenes was predominantly isolated from most animal samples, including fleece, fecal samples and claws, they were also frequently found in environmental samples such as litter and feed trough samples. Moreover, *L.innocua* was the majority species in roughage and roughage troughs, while *L.denitrificans* was mostly isolated from drinking water. In addition, all isolates from lymphnode samples were *L.ivanovii* (Table 4.8).

Table 4.8: *Listeria* species in different types of samples using biochemical tests

samples	sample	*Listeria* isolates	denitrificans	innocua	Ivanovii	monocytogenes	species
animal	fleece	7	1		1	3	2
	fecal sample	2				2	
	lymphnode	3			3		
	Claw	1				1	
	total	13	1		4	6	2
environmental	claw bath	0					
	feed	3		1		2	
	feed trough	2		1			1
	roughage	7	2	3		2	
	roughage trough	3		2		1	
	mineral stone	3	1	1		1	
	mineral powder	2		1		1	
	drinking water	4	2	1		1	
	waterpipeline insulation	1					1
	litter	13	1	2	1	8	1
	total	38	6	12	1	16	3
	grand total	51	7	12	5	22	5

4.1.3 *Escherichia coli*

4.1.3.1 *E.coli* positive samples

E.coli was isolated using selective media and biochemical tests as described in material and method. The overall E.coli prevalence was 60.8% (189/311), which was 62.7% (116/185) in animal samples and 57.9% (73/126) in environmental samples. Among animal samples, *E.coli* was predominantly isolated from fecal samples (97.1%, 68/70), followed by fleece samples (86.7%, 26/30) and lymphnodes (25.9%, 22/85).

Furthermore, environmental samples harbored *E.coli* in feed samples (56.5%, 13/23), feed containers (42.9%, 6/14), roughage (56.5%, 13/23), roughage containers (37.5%, 3/8), mineral stones (30.0% , 3/10), drinking water (47.83%, 11/23), waterpipeline insulation (100%, 1/1) and litter (100%, 22/22) (Table 4.9).

Table 4.9: *E.coli* positive number of samples

samples	sample	N	number of positive samples (%)
animal	fleece	30	26 (86.7)
	fecal sample	70	68 (97.1)
	lymphnode	85	22 (25.9)
	total	185	116 (62.7)
environmental	claw bath	1	0
	feed	23	13 (56.5)
	feed trough	14	6 (42.9)
	roughage	23	13 (56.5)
	roughage trough	8	3 (37.5)
	mineral stone	10	3 (30.0)
	mineral powder	1	0
	drinking water	23	11 (47.8)
	waterpipeline insulation	1	1 (100)
	litter	22	22 (100)
	total	126	73 (57.9)
	grand total	311	189 (60.8)

4.1.3.2 *E.coli* positive isolates using Polymerase Chain Reaction (PCR)

Confirmation of *E.coli* with PCR was performed. Of the 189 *E.coli* isolates, 179 (94.7%) were *E.coli* positive by PCR method (Table 4.10).

Table 4.10: Comparison between *E.coli* identification using conventional microbiological method with Polymerase Chain Reaction

sample		conventional microbiological isolation and identification	confirmation by PCR
animal	fleece	26	24
	fecal sample	68	67
	lymphnode	23	20
	total	117	111
environmental	feed	13	13
	feed trough	6	6
	roughage	13	13
	roughage trough	3	3
	mineral stone	3	3
	mineral powder	0	
	drinking water	11	11
	waterpipeline insulation	1	1
	litter	22	18
	total	72	68
	grand total	189	179

4.1.4 *Salmonella* identification

No sample was positive with any *Salmonella* spp.

4.1.5 *Yersinia enterocolitica* identification

No sample was positive with any *Yersinia enterocolitica*.

4.2 Quantification of bacteria

A total of 21 drinking water samples from 10 excursions was examined for Aerobic Plate Count (APC) and Enterobacteriaceae Count (EC). Mean APC (log CFU/ml) among these samples was 4.54, while mean EC (log CFU/ml) accounted to 2.03. The average of each excursion is described in Table 4.11.

Table 4.11: Aerobic Plate Count and Enterobacteriaceae Count in drinking water

excursion	N	Aerobic plate count (log CFU/ml)	Enterobacteriaceae count (log CFU/ml)
1	0	-	-
2	0	-	-
3	2	-	-
4	2	4.42	2.05
5	2	4.47	1.79
6	2	4.65	1.55
7	0	-	-
8	2	5.32	2.72
9	3	4.61	1.73
10	2	3.25	< 1.40
11	2	3.97	< 1.40
12	2	5.47	4.41
13	2	4.42	< 1.40
14	2	4.79	2.02

5. Discussion

5.1 Qualitative analysis (zoonotic agents)

5.1.1 Thermophilic *Campylobacter*

5.1.1.1 Campylobacter prevalence

In this study, all sample types from animals were positive with *Campylobacter*, most frequently in fecal samples (43.6%). Similar to our findings, *Campylobacter* was identified in 43.8% of rectal content at slaughterhouses in Great Britain (Milnes et al. 2008). In contrast, a lower prevalence in fecal samples was found in northeastern and southwestern Scotland (25%) (Rotariu et al. 2009). However, the prevalence of *Campylobacter* found in fecal samples was different, depending on sampling methods. A study in Lancashire revealed that the *Campylobacter* intestinal carriage at a slaughterhouse was higher (91.7%) than the shedding rate of *Campylobacter* in the environment (29.3%) from the same sheep herd (Stanley et al. 1998).

From other animal samples in this study, we obtained *Campylobacter* in fleece samples (20.0%) and in lymphnode samples (15.3%). In contrast to our findings, the prevalence in sheep pelts was 0% in southwest England (Small et al. 2002). The variation of these findings might depend on different factors, e.g., the cleanliness of fleece, number of *Campylobacter* burden in each herd and region, sampling procedures or laboratory methods. In addition, we also found *Campylobacter* in lymphnode samples. This type of sample was rarely investigated in other studies.

Among environmental samples, only litter, drinking water and feed were *Campylobacter* positive, with the highest positive percentage found in litter (40.9%). These samples were possibly contaminated with feces, which could be a source for positive results. Drinking water and feed were collected from troughs, which were near to the ground and where animals could step in. The average concentration of *Campylobacter* excreted in the environment was $3x10^4$ CFU/g (Ogden et al. 2009). Apart from such a direct fecal contact, flies found in sheep farms carried viable

Campylobacter and may contribute to transfer within and between groups of animals on farms, too (Sproston et al. 2010).

Table 5.1: *Campylobacter* species: Findings using conventional methods and PCR

sample		% of species					
		C.jejuni		*C.coli*		*C.lari*	
		conv.	PCR	conv.	PCR	conv.	PCR
animal	fleece	57.1	28.6	42.9		-	57.1
	fecal sample	58.8	8.3	35.3	47.2	5.9	38.9
	lymphnode	84.6	76.9	15.4		-	15.4
	total	64.8	26.7	31.5	30.4	3.7	35.7
environment	claw bath						
	feed	100	100	-		-	
	feed trough						
	roughage						
	roughage trough						
	mineral stone						
	mineral powder						
	drinking water	100	100	-		-	
	waterpipeline insulation						
	litter	66.7	66.7	33.3	11.1	-	11.1
	total	75	75	25	8.3	-	8.3
	grand total	66.7	35.3	30.3	26.5	3.0	30.9

* conv. = conventional methods

Using conventional methods, 3 species of thermophilic *Campylobacter* were identified (*C.jejuni*, *C.coli* and *C.lari*) in this study (Table 5.1). *C.jejuni* was predominantly isolated from both animal and environmental samples. Similarly, *C.jejuni* was the main species (90%) found in grazing sheep in Lancaster, United Kingdom, while *C.coli* was the next one (8%) and *C.lari* made up 2% of isolates (Jones et al. 1999).

With PCR, *C.jejuni* was also found most frequently in this study (Table 5.1), but in a lower rate (35.5%) compared to the conventional method (66.7%). In contrast to the

conventional approach, the percentage of *C.lari* (30.9%) was higher than *C.coli* (26.5%) (Table 5.1).

Among fecal samples, *C.coli* amounted to the highest percentage using PCR, following by *C.lari* (38.9%) and *C.jejuni* (8.3%). Similary, *C.coli* was in the majority among sheep fecal samples (47.4%) using PCR method, followed by *C.jejuni* (13.7%), *C.fetus* (12.3%) and *C.lari* (0.05%) (Grove-White et al. 2010).

5.1.1.2 Epidemiology of Campylobacter

Source of Campylobacter

In this study, Pulsed Field Gel Electrophoresis (PFGE) was carried out in order to trace sources and transfer routes of *Campylobacter*. Excursions represented different sheep farms in the same region (14 excursions to 9 sheep farms). Within individual sheep herds (vertical direction - Table 4.6), PFGE-patterns were found to be identical in both animal and environmental samples in the same farm (7^{th}, 9^{th} and 12^{th} excursion). In contrast, PFGE-patterns obtained from the animals and environmental samples were not identical in samples from the 8^{th} excursion.

Comparing the individual sheep farms (horizontal lines in Table 4.6), the same PFGE-types or subtypes were identified also in the different farms. For example, PFGE-type K-6 was found at the occasions of 6^{th}, 7^{th} and 9^{th} excursions. We concluded that *Campylobacter* isolated in different farms carried the same PFGE–types, indicating links between the farms. Another explanation could be the microbiological technique, i.e., the restriction enzyme used for this purpose. The use of another additional enzyme should clarify this important observation.

Importance of Campylobacter in sheep

In this study, *C.jejuni*, *C.coli* and *C.lari* were identified. In Austria, *C.jejuni* was the major species causing gastroenteritis in humans (Much et al. 2009). In Germany, all 3 species (*C.jejuni*, *C.coli* and *C.lari*) were found to cause illness, but C.*jejuni* was

mostly isolated (70.8 %) among patients (RKI 2009). However, the number of finally identified species was different depending on conventional or molecularbiological procedures, which might be a methodological question (5.1.1.3).

5.1.1.3 Laboratory methods

Cultural techniques provide the advantage of isolates for further identification or typing, but here some difficulties were observed. Using conventional methods, mixed cultures cannot be identified, because of 2 isolates might aggregate together (Miller et al. 2000), resulting possibly in contradiction. In addition, the interpretation of hippurate hydrolysis is difficult (i.e., separation between weak positive and negative), if the color is not clear enough. Finally, emerge of resistance of *Campylobacter* to nalidixic acid has been reported (Bostan et al. 2009; Rahimi et al. 2010). Consequently, to differentiate between *C.lari* from resistant *C.coli* using biochemical test could be ambiguous.

Simutaneously, in this study, Polymerase Chain Reaction (PCR) was applied to the *Campylobacter* isolates in order to confirm the genus. Here, 92.6% of *Campylobacter* isolates obtained by isolation and identification were positive with PCR technique, too. We used the primers designed from 23S rRNA to identify the genus *Campylobacter*, which was described by Wang *et al.* (2002). Primers used for *C.jejuni* and *C.coli* identification were obtained from the same authors, while primers for *C.lari* were taken over from Eyers *et al.* (1993). For PCR identification of *C.jejuni* and *C.coli*, we used the primers designed by Wang et al. (2002). These authors used these primers as one of 6 pairs in a multiplex PCR. For this multiplex PCR, the sensitivity ranged from 10^8 to 10^{13} for *C. jejuni* and from 10^6 to 10^{13} for *C. coli* CFU per ml (Wang et al. 2002).

As described in Table 5.1, our results for species identification using conventional technique and PCR were different. The conventional method used in this study followed the standard procedure, difficulties have been described already. The combination of conventional and molecular techniques could add to the information value of *Campylobacter* confirmation, until this problem has been solved.

5.1.2 *Listeria* spp.

5.1.2.1 Listeria prevalence

In this study, *Listeria* was isolated from both animal and environmental samples. Fecal samples (2.6%), fleece (20%), lymphnodes (3.5%) and claw swabs (50%) were positive for "animal type" samples. Higher prevalence (sheep and goat) was described in farms with a history of listeriosis in New York State (32.9%), while farms without history from the same region had a lower percentage (5.9%) (Nightingale et al. 2004).

A high contamination rate was found among environmental samples, *Listeria* was isolated from litter (59.1%), mineral stones (30%), roughage (26.1%), roughage troughs (25%), drinking water (17.4%), feeding troughs (14.3%) and feed samples (8.7%). Silage samples were found to be contaminated with *L.monocytogenes* during an outbreak of clinical listeriosis in a sheep farm in Austria (Wagner et al. 2005).

Not only *L.monocytogenes* was identified in this study, but also *L.denitrificans*, *L.innocua* and *L.ivanovii*. *L.innocua* (3.6%) was also isolated from healthy sheep in India (Yadav and Roy 2009). At retails in Brisbane, *L.innocua* was identified from lambs (24%) (Ibrahim and Mac Rae 1991).

5.1.2.2 Epidemiology of Listeria spp.

Source of Listeria

From our study, *Listeria* spp. was more frequently found in environmental samples (27%) than in samples from animals (6.2%). So, the environment could be a source of *Listeria* in sheep farms. Indeed, an outbreak of clinical listeriosis in sheep was caused by feeding grass silage, which was contaminated with 5 $\log_{10}$ CFU *L.monocytogenes*/g (Wagner et al. 2005).

L.monocytogenes ribotypes found in clinical cases and the respective fecal samples were more frequently identified in environmental than in feed samples, suggesting that infected animals may shed *L.monocytogenes* into the farm environment (Nightingale et al. 2004).

Importance of Listeria in sheep

Among all samples in this study, *L.monocytogenes* was the most frequently isolated species within the genus *Listeria*. *L.monocytogenes* is an important foodborne pathogen of human listeriosis (Doyle and Beuchat 2007). It was found along the lamb production chain, including sheep carcass samples at a slaughterhouse in Turkey (5%) (Akpolat et al. 2004) and samples from lambs (40%) at retail markets in northern Bristol (MacGowan et al. 1994).

5.1.2.3 Laboratory diagnosis of Listeria spp.

In this study, *Listeria* isolation and identification followed the standard method (ISO 11290:1995), 4 species (*L.monocytogenes*, *L.denitrificans*, *L.innocua* and *L.ivanovii*) were identified. Using biochemical tests, 5 of 51 isolates could not be identified as for their species. Thus, the selective culture method was effective in order to provide identification results.

With regard to foodborne human health aspects, it is important to focus on *L.monocytogenes* in order to understand the epidemiology through the lamb production chain. Other laboratory diagnosis, such as serotyping and PFGE, should be applied to provide more information on the epidemiology of the agent (Esteban et al. 2009; Gianfranceschi et al. 2009). This study did not include this technique for *Listeria*, the importance of *L.monocytogenes* within the lamb food chain was stressed as such.

5.1.3 *Escherichia coli*

5.1.3.1 E.coli prevalence

Camparing all sample types in this study, *E.coli* was identified at a different rate. Among animal samplers, fecal samples were most frequently found to be positive (97.1%), followed by fleece (86.7%) and lymphnodes (25.9%). Most studies on sheep farms focused on enteropathogenic *E.coli*. The herd prevalence of *E.coli* O157:H7 was 8.7%, while the individual prevalence was 7.3% (Oporto et al. 2008). In northeastern Scotland, the percentage of individuals shedding *E.coli* O157 was 5.8%, whereas the prevalence at farm level was 42.8% with an amount of 10^2 CFU/g (Solecki et al. 2009). In the southwestern United States, fecal and wool samples carried *E.coli* O157:H7 in a percentage of 9% and 18%, respectively (Edrington et al. 2009).

5.1.3.2 Laboratory diagnosis of E.coli

Fluorocult® ECD Agar was used for isolation and identification. This medium is a selective medium used for the *E.coli* examination in meat, meat products and food, recommended in standard prescriptions (such as ISO 10110 and ISO 6391:1996). Its ability to inhibit competing microflora by a bile salt , fluorescence under UV light and a positive indole reaction make it convenient and simply to do.

Fluorescence on ECD agar combined with positive indole reaction of resulted in a specificity of 99.2% and a sensitivity of 85.8% (Heizmann et al. 1988). Most *E.coli* contain the enzyme β-glucuronidase. This enzyme transfers 4-methylumbelliferyl-β-Dglucuronide (MUG) into glucuronic acid and methylumbelliferon, the latter one fluorescing under UV light. Using β-D-glucuronidase activity as an aid of selection, only 61.1% of VTEC could be identified (Beutin et al. 1994). In addition, *E.coli* O157:H7 do not have any β-D-glucuronidase activity (Feng and Lampel 1994; Monday et al. 2001).

E.coli isolates obtained from this study were fluorescent positive from Fluorocult® ECD Agar, meaning a certain percentage of enteropathogenic *E.coli* was excluded by the condition of the method. For an internal test, we tested the strains for a few virulence genes (*hyl*, *vt1*, *vt2*, and *eae*) (Fries et al. 2005). Results revealed that less than 3% of the isolates from Fluorocult® ECD Agar carried virulence genes (Table 5.2). However, the real prevalence in this study should be higher with referred to out methods, which must be taken into the consideration.

Table 5.2: Virulence genes in *E.coli* isolates from samples

virulent genes	n	positive
hyl	110	3
vt-1	110	3
vt-2	110	2
eae	110	2

In comparison, using molecular methods (PCR), 94.7% of *E.coli* isolates obtained from our samples were identified as *E.coli*. The primer used in this reaction had been derived from the "universal stress protein A *(uspA)*", which had been described by Osek et al. (2000).

5.1.4 *Salmonella*

5.1.4.1 Salmonella prevalence

All samples from animals and from the environment were *Salmonella* negative, which corroborates results from other studies. The prevalence of *Salmonella* reported from such studies was low in sheep fecal samples, ranging from 1.1% in Great Britain (Milnes et al. 2008) to 7% in United States (Edrington et al. 2009). In contrast, its prevalence in fleece varied from 7.8% (Small et al. 2002) to 50% (Edrington et al. 2009)

5.1.4.2 Laboratory diagnosis of Salmonella

The identification of *Salomonella* followed the standard method ISO 6579:2002. Two selective medium were used, Rambach® (Merck) and Brilliant-green-Phenol-red-Lactose-Sucrose agar (BPLS). Rambach agar (Merck) is recognized as a reliable and selective diagnostic medium for the identification of *Salmonella* species (Garrick and Smith 1994; Carrique-Mas et al. 2009), being more effective than Xylose-Lysine-Desoxycholate (XLD) agar from both raw and ready-to-eat products (El-Sherif and Elmossalami 1998). Using the same pre-enrichment and enrichment media and procedures as was used in our study, the detection limit of *Salmonella abortus ovis* in ovine abortion was 6.5 x 10^3-6.5 x 10^4 bacteria (Sting et al. 1997). As *Salmonella* was not identified in any type of sample in our study, the prevalence of *Salmonella* among sheep herds or the number of this pathogen may actually be low. Serological tests may be useful to provide more data for this argument.

5.1.5 *Yersinia enterocolitica*

5.1.5.1 Yersinia enterocolitica prevalence

Y.enterocolitica was not obtained in this investigation. The prevalence of *Y.enterocolitica* in sheep has been described earlier: isolation rates from intestines at slaughterhouses ranged from 8.0% (Milnes et al. 2008) to 10.7% (McNally et al. 2004). Evidence for the association between *Y.enterocolitica* infection in humans and this pathogen in sheep was rarely reported. A study in Great Britain found some pathogenic biotypes from sheep at a slaughterhouse (McNally et al. 2004), while another did not report any pathogenic strains from sheep (Bucher et al. 2008).

5.1.5.2 Laboratory diagnosis of Yersinia enterocolitica

For isolation and identification, the standard method (ISO 10273:2003) was applied. The duration of the enrichment in this procedure was regarded to be not optimal for the isolation of pathogenic *Y.enterocolitica* from pig tonsils (Van Damme et al.

2010). Reducing the enrichment time in PSB from 5 to 2 days resulted in a significantly higher recovery rate (94.2%) in swine (Van Damme et al. 2010). Moreover, studies among food samples revealed that the efficacy of the enrichment procedure depends on the bio/serotypes of *Yersinia* spp. and on the type of food to be examined. Hence, the use of more than one medium for both enrichment and plating should be appropriate and might result in higher recovery rates of *Yersinia* spp. (de Boer 1992).

5.2 Quantitative and qualitative analysis for drinking water samples

A total of 23 drinking water samples was collected in this study, 21 samples from 10 excursions were tested for APC and Enterobacteriaceae count (EC). The mean APC (log_{10} CFU/ml) was 4.54. Following a public statement of Federal Ministry of Food, Agriculture and Consumer Protection, Germany (BMELV), the APC of drinking water for animals should not exceed 1,000 CFU/ml at 37°C and 10,000 CFU/ml at 20°C (BMELV 2007). Our results showed that mean APC of drinking water was higher than recommended at 37°C, and lower than that number at 20°C. However, we did not record the temperatures at the site of collection.

BMELV also states that drinking water should be free from *Salmonella* and *Campylobacter* (in 100 ml) and *E.coli* (10 ml). In this study, *Campylobacter* was identified in 8.7% (2/23) from 10 ml drinking water, while all of them were free from *Salmonella* (in 10 ml). The isolation rate of *E.coli* was 47.8% (11/23) from a sample unit of 10 ml. Our study found that drinking water samples did not contain any *Salmonella*, while *Campylobacter* and *E.coli* were isolated. Therefore, *Campylobacter* and *E.coli* should be concerned more seriously as a hygienic parameter for drinking water in animal farming.

6. Summary

Zoonotic Agents in Sheep Farms in Brandenburg, Germany

The aim of this study was to indicate the prevalence of thermophilic *Campylobacter, Listeria* spp., *Escherichia coli*, *Salmonella* spp. and *Yersinia enterocolitica* in a sheep food chain in Brandenburg.

The study was performed from July 2008 to August 2009. A total of 321 samples was collected from 9 sheep farms, supplying their animals to the same slaughterhouse. Samples were examined using qualitative (for thermophilic *Campylobacter, Listeria, E.coli, Salmonella* and *Yersinia*) and quantitative methods (Aerobic Plate Count and Enterobacteriaceae Count for investigation of drinking water samples).

Thermophilic *Campylobacter*, *Listeria* spp. and *Escherichia coli* were identified from animal and environmental samples, with an average of 20.4%, 14.3% and 60.8%, respectively. *Salmonella* spp. and *Yersinia enterocolitica* were not found in any sample.

Campylobacter was most frequently identified in fecal samples (43.6%). Using conventional techniques, *C.jejuni* was predominantly found (66.7%), followed by *C.coli* (30.3%) and *C.lari* (3.0%). Using PCR, the majority of *Campylobacter* species was *C.jejuni* (35.3%), followed by *C.lari* (30.9%) and *C.coli* (26.5%).

The isolation rate of *Listeria* spp. was high in environmental samples (27%), whereas the percentage of animal samples accounted to 6.2%. The majority of *Listeria* isolates was *L.monocytogenes* (43.1%), while *L.innocua*, *L.denitrificans*, and *L.ivanovii* were found in a percentage of 23.5%, 13.7% and 9.8%, respectively.

Among animal samples, *E.coli* was predominantly isolated from fecal samples (97.1%). Among environmental samples, litter was the most contaminated sample type (100% positive).

Campylobacter strains were in some farms identical both in animal and environmental samples (7^{th}, 9^{th} and 12^{th} excursion), which was discovered using PFGE techniques. Comparing the individual sheep farms, the same PFGE-types or subtypes were obtained also from different farms (PFGE-type K-6 in 6^{th}, 7^{th} and 9^{th} excursion).

A total of 23 drinking water samples was collected, of them, 21 samples from 10 excursions were tested for Aerobic Plate Count (APC) and Enterobacteriaceae Count (EC). The mean APC (log_{10} CFU/ml) was 4.54. *Campylobacter* was identified in 8.7% (2/23) of drinking water samples, while all of them were free from *Salmonella* (in 10 ml). The *E.coli* isolation rate was 47.8% (11/23) in these samples.

In conclusion, thermophilic *Campylobacter*, *Listeria* spp. and *Escherichia coli* were identified in both animal and environmental samples, while *Salmonella* spp. and *Yersinia enterocolitica* were not found. Shedding of *Campylobacter* via feces deems to play an important role with regard to the distribution of this pathogen in sheep herds. For *Listeria*, a high contamination rate was found in the environment, which may play a role for this agent. Finally, the species identification of *Campylobacter* differs depending on the techniques: conventional techniques and PCR techniques revealed different results.

7. Zusammenfassung

Zoonose Erreger in Schafhaltungsbetrieben in Brandenburg (Deutschland)

Ziel dieser Studie war es, die Prävalenz von thermophilen *Campylobacter, Listeria* spp., *Escherichia coli, Salmonella* spp. und *Yersinia enterocolitica* in Schafhaltungsbetrieben in Brandenburg zu untersuchen.

Die Untersuchungen wurden im Zeitraum von Juli 2008 bis August 2009 durchgeführt. Insgesamt wurden 321 Proben von neun Betrieben, die denselben Schafschlachtbetrieb beliefern, gewonnen. Die Proben wurden qualitativ untersucht (thermophile *Campylobacter, Listeria* spp., *Escherichia coli, Salmonella* spp. und *Yersinia enterocolitica),* ein quantitativer Nachweis (Gesamtkeimzahlbestimmung und Bestimmung der Enterobacteriacae) erfolgte bei der Untersuchung von Trinkwasser.

Thermophile *Campylobacter*, *Listeria* spp. und *Escherichia coli* traten im Durchschnitt bei 20,4%, 14,3% und 60,8% bei Tier- und Geländeproben auf. *Salmonella* spp. und *Yersinia enterocolitica* wurden nicht gefunden.

Campylobacter konnte hauptsächlich in Kotproben nachgewiesen werden (43,6%). Auf konventioneller Basis war *C.jejuni* vorherrschend (66,7%), gefolgt von *C.coli* (30,3%) und *C.lari* (3,0%). Die PCR-Untersuchung ergab als Hauptvertreter *C.jejuni* (35,3%), gefolgt von *C.lari* (30,9%) und *C.coli* (26,5%).

Die Nachweisrate von *Listeria* spp. lag bei den Umweltproben mit 27% höher als bei den Tierproben mit 6,2%. Der Hauptanteil der Isolate wurde als *L.monocytogenes* (43,6%) identifiziert. *L.innocua, L.denitrificans und L.ivanovii* machten jeweils 23,5%, 13,7% und 9,8% aus.

Bei den Tierproben wurde *E.coli* am häufigsten bei den Kotproben gefunden (97,1%), während *E.coli* unter den Umweltproben vor allem bei Tretmist auftrat (100%).

Innerhalb der einzelnen Schafherden waren manche PFGE-Muster in Tier- und Umweltproben identisch (7., 9. und 12. Probenahme). Im Vergleich der einzelnen Betriebe konnten identische PFGE-Typen oder –Subtypen in drei verschiedenen Betrieben identifiziert werden (PFGE-Typ K-6 bei Probennahme 6, 7 und 9).

Insgesamt wurden 23 Trinkwasserproben genommen, von denen 21 Proben (10 Probenahmen) auf die Gesamtkeimzahl (APC) und den Gehalt von Enterobacteriaceae (EC) untersucht wurden. Die durchschnittliche GKZ betrug ($\log_{10}$ KbE/ml) 4,54. *Campylobacter* wurde in 8,7% (2/23) der Trinkwasserproben nachgewiesen, während alle Proben frei von *Salmonella* waren (in 10 ml). Die Isolationsrate von *E.coli* betrug 47,8% (11/23).

Insgesamt wurden in dieser Untersuchung thermophile *Campylobacter*, *Listeria* spp. und *Escherichia coli*, dies in Tier- als auch in Unweltproben, nachgewiesen. *Salmonella* spp. und *Yersinia enterocolitica* wurden nicht gefunden. Die Ausscheidung von *Campylobacter* mit den Fäzes kann bei der Verbreitung innerhalb von Schafherden eine Rolle spielen kann. In der Umwelt wurde eine hohe Kontaminationsrate von *Listeria* gefunden, was bei diesem Erreger eine Rolle spielen kann. Die Untersuchungstechnik (konventionelle im Vergleich zur PCR-Technik) hat Einfluss auf die Spezies-Verteilung von *Campylobacter*.

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9. Appendix

9.1 General biological analysis

9.1.1 Gram staining

Preparation of the slide

1. Place one drop of NaCl solution on the glass slide.
2. Take a little mass of the suspected colony and mix with NaCl using a loop.
3. Let the slide dry by air.
4. Fix the slide by slowly flaming from underneath of the slide 3 times.

Gram staining

1. Completely cover the slide with crystal violet solution for 1 minute.
2. After pouring off the solution, wash the slide with Lugols' solution.
3. Completely cover the slide with Lugols' solution for 1 minute.
4. Rinse the slide with distilled water for 5 seconds and be careful to wash off all the substance.
5. Dip the slide in absolute alcohol (96% w/v) until all color is lost.
6. Rinse the slide with distilled water for 5 seconds and be careful to wash off all substance.
7. Completely cover the slide with Safranin solution for 1 minute.
8. After pouring off the solution, rinse the slide with distilled water for 5 seconds and be careful to wash off all substance.
9. After air drying, inspect this slide under light microscope.

Analysis

1. Cover the area with immersion oil.
2. Inspect under x100 magnification.
3. Record the color (red or blue), shape and aggregation of the cells on the slide.

9.1.2 Oxidase test

Oxidase test was performed using a commercial test strip (Bacident®; Merck). A single and pure culture was taken directly on the test strip using a loop. Within 60 seconds, positive result developed blue or violet blue on the test strip. In contrast, there was no color change in case of a negative result.

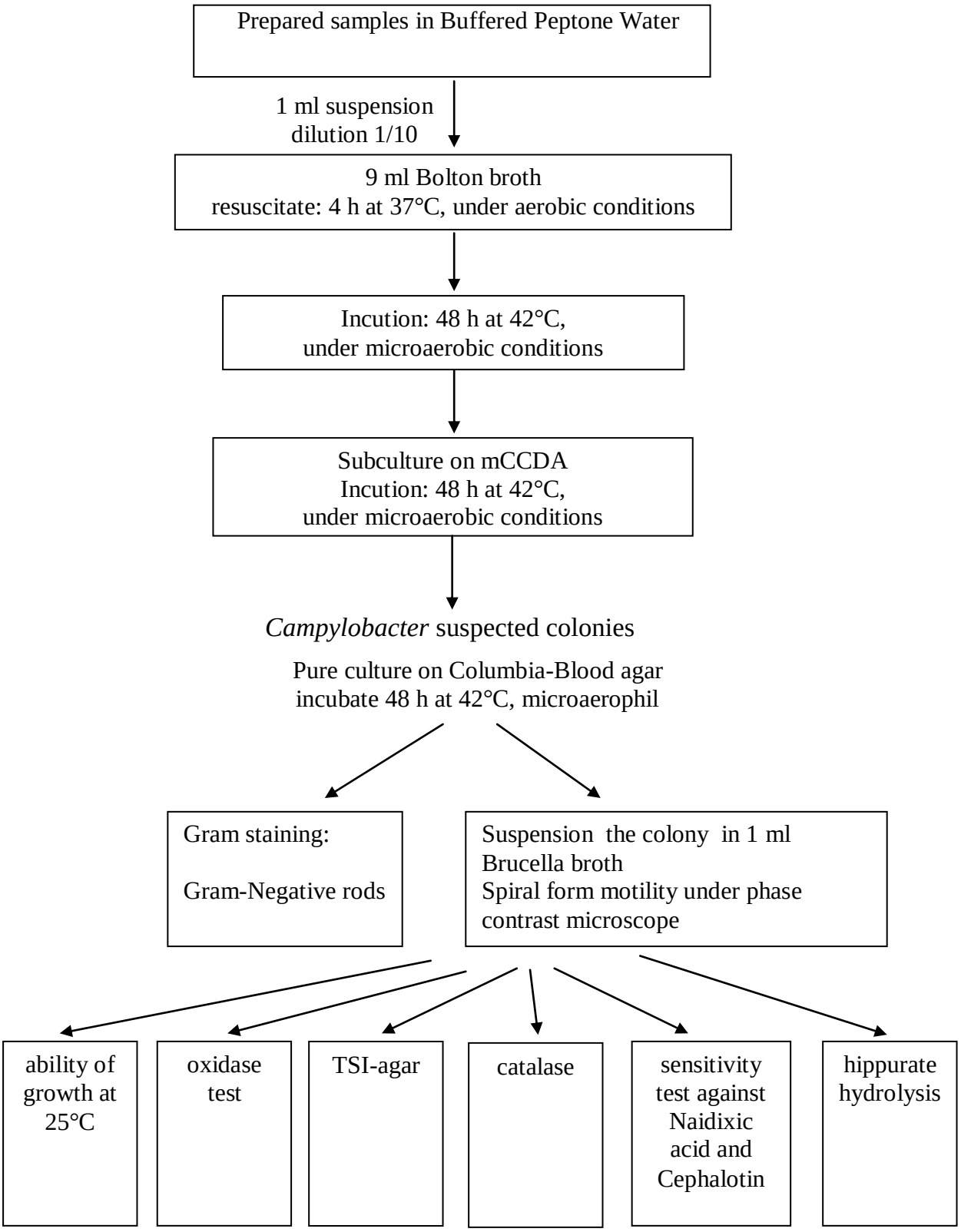
9.1.3 Catalase test

A Culture from standard I agar (Merck®) was used for catalase test in order to avoid false positive ones from blood agar. The suspected colony was taken on a clean glass slide. 3% H_2O_2 solution was dropped on the bacterial mass. Positive results showed the formation of bubbles.

9.2 Identification and differentiation of zoonotic agents

9.2.1 Identification of thermophilic *Campylobacter*

9.2.1.1 Isolation of *Campylobacter* spp.



9.2.1.2 The laboratory form of *Campylobacter* identification

Untersuchung: auf Campylobacter

Aufarbeitung im Labor:

Probenentnahme-Datum: Material der Probe:

Reaktionsmuster für Campylobacter spp.					1	2	3	4	5	6	7	8	9	10
Labor Nr.	*C. jejuni* DSM 4688	*C. coli* DSM 4689	*C. lari* DSM 1375	*C. upsa-liensis*										
CCDA														
GRAM	Gramnegativ Zarte, gekrümmte Stäbchen													
Katalase	+	+	+	-/(+)										
Oxidase	+	+												
Bewegl. hä. Tropfen	+	+												
25° C Wachstum	-	-	-	-										
Glukose	-	-	-	-										
Saccharose	-	-	-	-										
Laktose	-	-	-	-										
Gasbildung	-	-	-	-										
H_2S	-	-	-	-										
Hippurat-H.	+	-	-	-										
Nalidixins. (30 µg)	S	S	R	S										
Cephalotin (30 µg)	R	R	R	S										
Stereomikrosk.														
Auswertung														
Stamm Gesammelt														

KW: Kein Wachstum auf der Platte

KV : Platte bewachsen aber keine verdächtigen Kolonien

CV : *Campylobacter*-verdächtige Kolonie

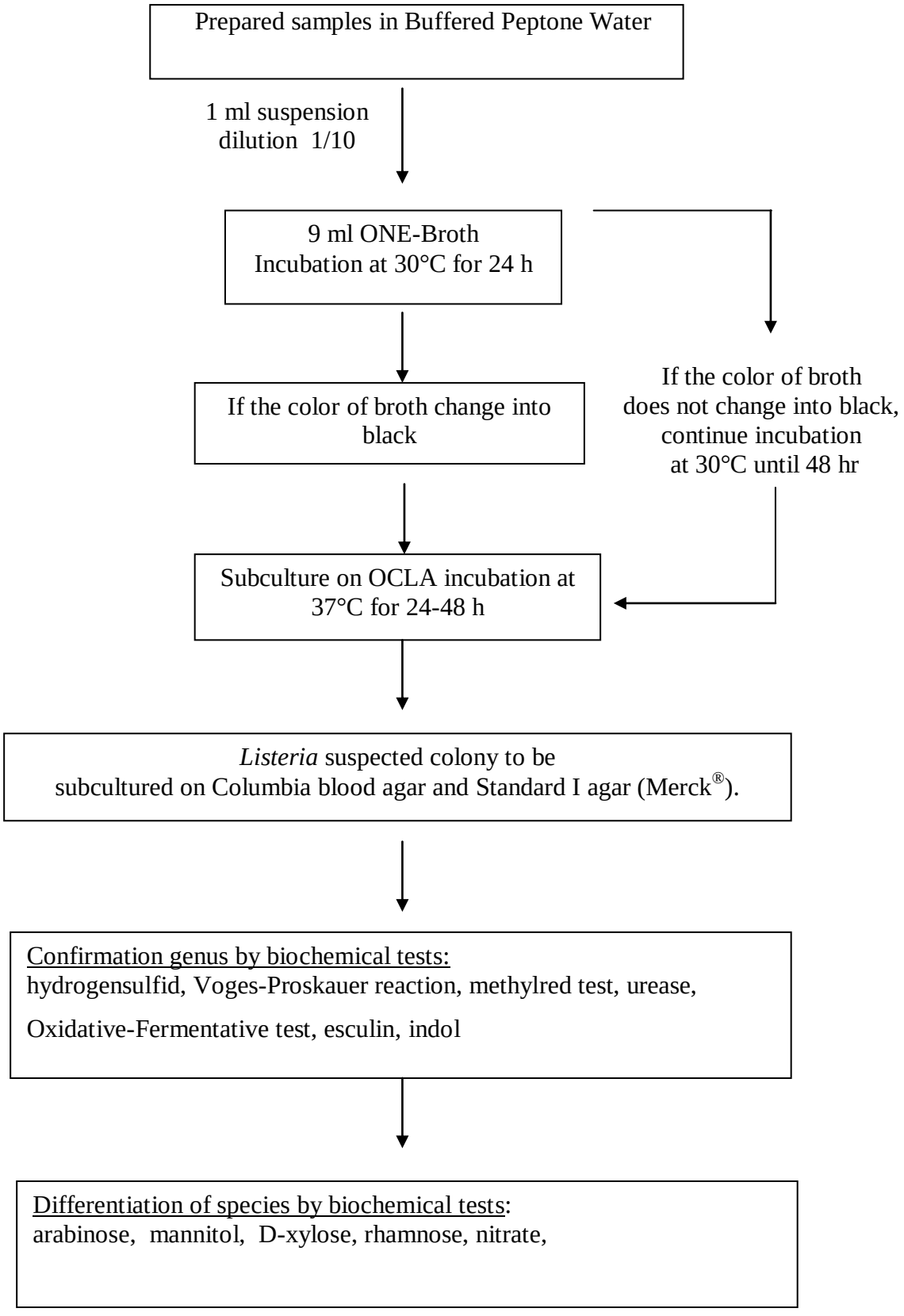
? : unklar

Positiv-Kontrolle: *Campylobacter coli* DSM 4689 und/oder *Campylobacter jejuni* DSM 4688

Negativ-Kontrolle: *E.coli* ATCC 25922

9.2.2 Identification of *Listeria*

9.2.2.1 Isolation of *Listeria* spp.



9.2.2.2 The laboratory form for *Listeria* identification

Untersuchung: *Listerien*

Aufarbeitung im Labor:

Probenentnahme-Datum: Material der Probe:

Labor Nr.	Selektive Anreicherung: ONE-Bouillon 1 ml Untersuchungs-Material in 9 ml ONE-Bouillon (1:10)		OCLA 10 µl aus der geschüttelten Probe entnehmen und auf OCLA ausstreichen		Listeria- verdächtige Kolonien Blaue Kolonien mit bez. ohne Präzipitationszone	Stamm Gesammelt
	Bei 30°C für 24 +/- 2 Std	Bei 30°C für 48 +/- 2 Std	Bei 37°C für 24 +/- 2 Std inkubieren Negative Platten nochmals 24 Std.			
1			24 Std.:	48 Std.:		
2			24 Std.:	48 Std.:		
3			24 Std.:	48 Std.:		
4			24 Std.:	48 Std.:		
5			24 Std.:	48 Std.:		
6			24 Std.:	48 Std.:		
7			24 Std.:	48Std.:		
8			24 Std.:	48 Std.:		
9			24 Std.:	48 Std.:		
10			24 Std.:	48 Std.:		
11			24 Std.:	48 Std.:		
12			24 Std.:	48 Std.:		
13			24 Std.:	48 Std.:		
14			24 Std.:	48 Std.:		
15			24 Std.:	48 Std.:		

KW: Kein Wachstum auf der Platte
KV : Platte bewachsen aber keine verdächtigen Kolonien
LV : *Listeria*-verdächtige Kolonie
? : unklar
+ : Schwarzfarben von ONE bouillon
- : kein schwarzfarben von ONE bouillon

ıv-Kontrolle: *Listeria monocytogenes* DSM 20600
ıtiv-Kontrolle: *Enterococcus faecalis* DSM 2570

9.2.2.3 The laboratory form for *Listeria* biochemical test

Biochemie für *Listeria* spp.

Aufarbeitung im Labor: Biochemie-Datum:

Probenentnahme-Datum: Material der Probe:

Reaktionen	*L.monocytogenes* DSM 20600	1	2	3	4	5	6	7	8	9	10
Mikroskopie	Grampositiv Klien, gerade Stäbchen										
Beweglichkeit: 25°C	+										
Katalase	+										
Oxidase	-										
VPR / MR	(+) / +										
Urease	-										
O/F - Test (Glucose)	+ / +										
Esculin	+										
Indol	-										
H_2S	-										
Auswertung											

9.2.2.4 The laboratory form for *Listeria* spp. differentiation

Biochemie für die Spezies-Differenzirung: *Listerien*

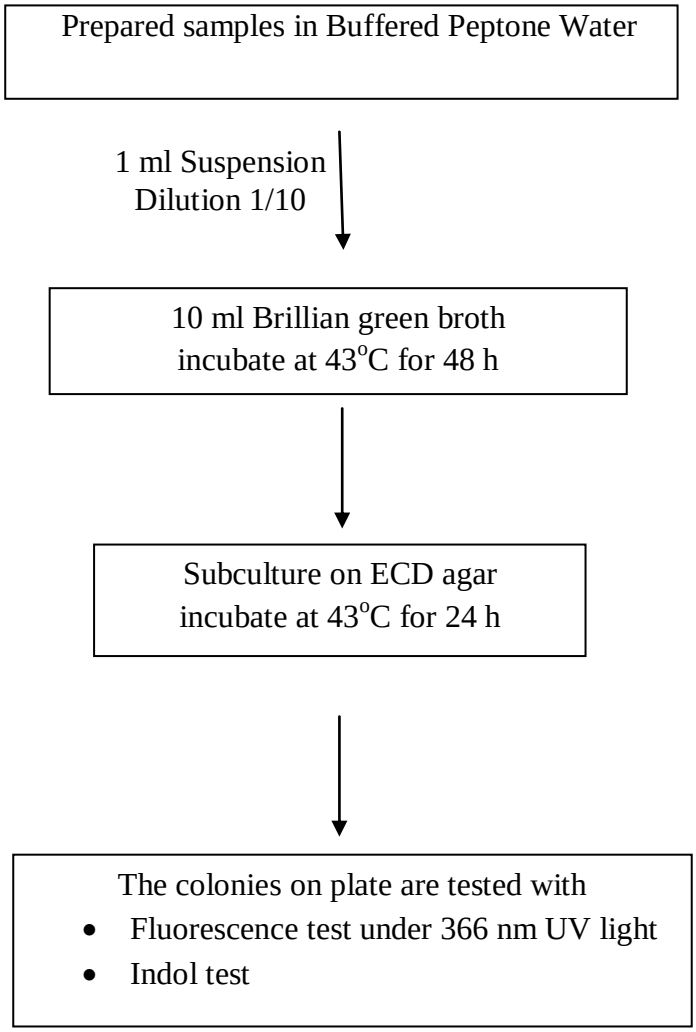
Aufarbeitung im Labor: Biochemie-Datum:

Probenentnahme-Datum: Material der Probe:

Listeria	monocy-togenes	innocua	seeligeri	welsh-imeri	ivanovii	grayi	murrayi	denitri-ficans	1	2	3	4	5	6	7	8	9	10
Vollstäandige	+	-	+	-	+	-	-	-										
Nitrat	-	-	-	-	-	-	+	+										
CAMP																		
* St.aureus	+	-	+	-	-	-	-	-										
* Rh.equi	-	-	-	-	+	-	-	-										
Rhamnose	+	D	-	D	-	-	D	-										
D-Xylose	-	-	+	+	+	-	-	+										
Arabinose	-	-			-	-	-	+										
Mannitol	-	-	-	-	-	+	+	-										
VPR	+	+	+	+	+	+	+	-										

9.2.3 Identification of *E.coli*

9.2.3.1 Isolation of *E.coli*



9.2.3.2 The laboratory form for *E.coli* identification

Untersuchungs: auf *E. coli*

Aufarbeitung im Labor:

Probenentnahme-Datum: Material der Probe:

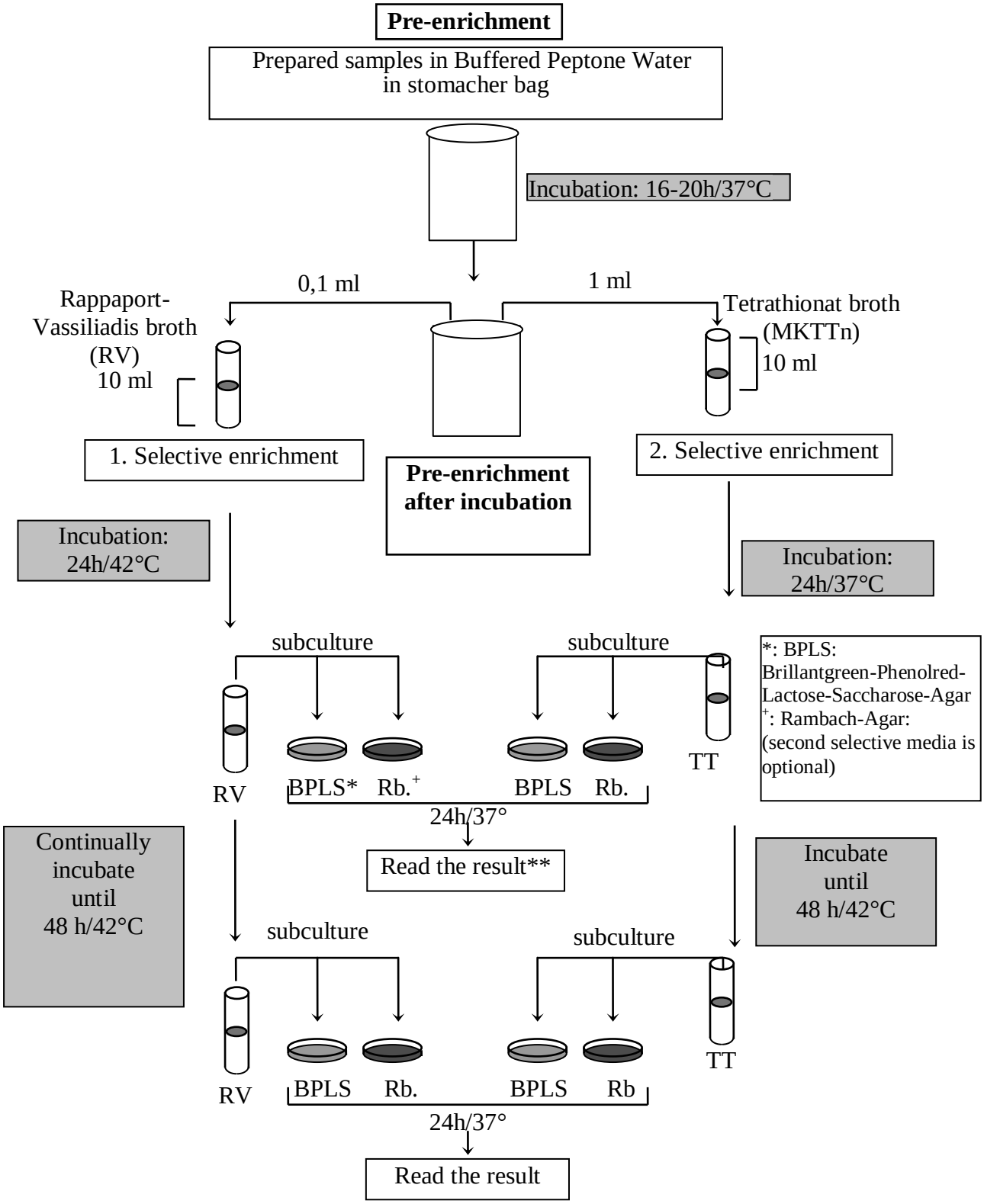
Lfn. Nr.	Labor-Nr	Brila-Anreicherung 48 Stunden/43°C			Ergebnis	Stamm Gesammelt
		ECD-Agar (24 Stunden / 44˚C)	Fluoreszenz 366 nm	Indol bildung		
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						

W: Kein Wachstum auf der Platte
KV : Platte bewachsen aber keine verdächtigen Kolonien
EV : *E.coli* -verdächtige Kolonie
? : unklar

Positiv-Kontrolle: *E.coli* ATCC 25922
Negativ-Kontrolle: *Staphylococcus aureus* DSM 799

9.2.4 Identification of *Salmonella*

9.2.41.1 Isolation of Salmonella



9.2.4.2 The laboratory form for Salmonella identification

Untersuchungen auf *Salmonella*
Aufarbeitung im Labor:
Datum der Probenentnahme: Material der Probe:

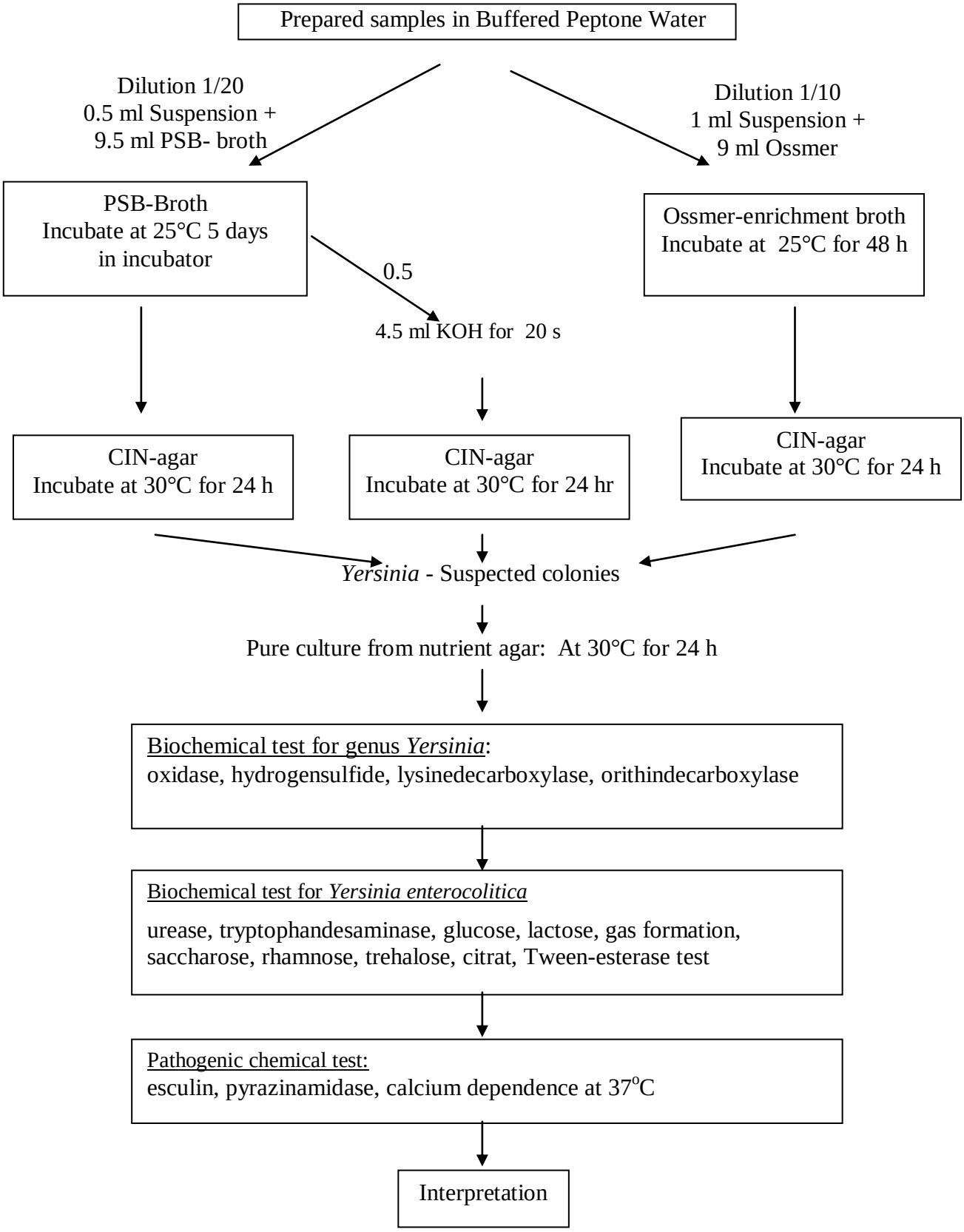
Labor Nr.	24 Stunden				48 Stunden				Salmonella-Agglutination				Ergebnis Nach Screening Genus Salmonella	Stamm Gesammelt
	Rambach		BPLS		Rambach		BPLS		Subkultur	Nach Subkultivierung				
	RV	TT	RV	TT	RV	TT	RV	TT	ST I	Poly I	Poly II	NaCl		
1														
2														
3														
4														
5														
6														
7														
8														
9														
10														
11														
12														
13														
14														
15														

KW: Kein Wachstum auf der Platte
KV : Platte bewachsen aber keine verdächtigen Kolonien
SV : *Salmonella*-verdächtige Kolonie
? : unklar

Positiv-Kontrolle:*Salmonella typhimurium* DSM 5569
Negativ-Kontrolle:*E.coli* ATCC 25922

9.2.5 Identification of pathogenic *Yersinia enterocolitica*

9.2.5.1 Isolation of *Yersinia* spp.



9.2.5.2 The laboratory form for *Yersinia* identification

Untersuchung: auf *Yersinia*

Aufarbeitung im Labor:

Probenentnahme-Datum: Material der Probe:

Labor Nr.	1 ml Suspension zu 9 ml Oßmer, Anreicherung 48 h 25°C Brutschrank 0,5 ml Susp. zu 9,5 ml PSB, Anreicherung 5 Tg im 25° Brutschrank			Subkultur von-verdächtigen Kolonien auf ST I 30°C Brutschrank	Ergebnis	Stamm Gesammelt
	Aus Oßmer 25°C/48h auf CIN 30°C/24-48 h	Aus PSB ohne KOH auf CIN 30°C/24-48 h	Aus PSB mit KOH auf CIN 30°C/ 24-48 h			
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						

KW: Kein Wachstum auf der Platte

KV : Platte bewachsen aber keine verdächtigen Kolonien

YV : *Yersinia*-verdächtige Kolonie

? : unklar

Positiv-Kontrolle: *Yersinia enterocolitica* DSM 4780

Negativ-Kontrolle: *E.coli* ATCC 25922

9.2.5.3 The laboratory form for *Yersinia enterolocolotica* biochemical tests

Biochemie für *Yersinia enterolocolitica*

Aufarbeitung im Labor: Biochemie-Datum:

Probenentnahme-Datum: Material der Probe:

Merkmale *Yersinia*	*Yersinia*	1	2	3	4	5	6	7	8	9	10
Mikroskopie	Gramnegativ Stäbchen am ende oft Körnchen										
Hydrogensulfid	-										
Oxidase	-										
Lysindecarboxylase	-										
Ornithindecarboxylase	+										
Harnstoff	+										
Tryptophandesaminase	-										
Glucose	+										
Gasbildung aus glucose	-										
Lactose	-										
Saccharose	+										
Rhamnose	-										
Citrat	-										
Trehalose	-/+										
Tween-Esterase Test	-/+										
Äskulin	-										
Pyrazinamidase	-										
Calciumabhängigkeit bei 37^{o}C	+										
Ergebnis											

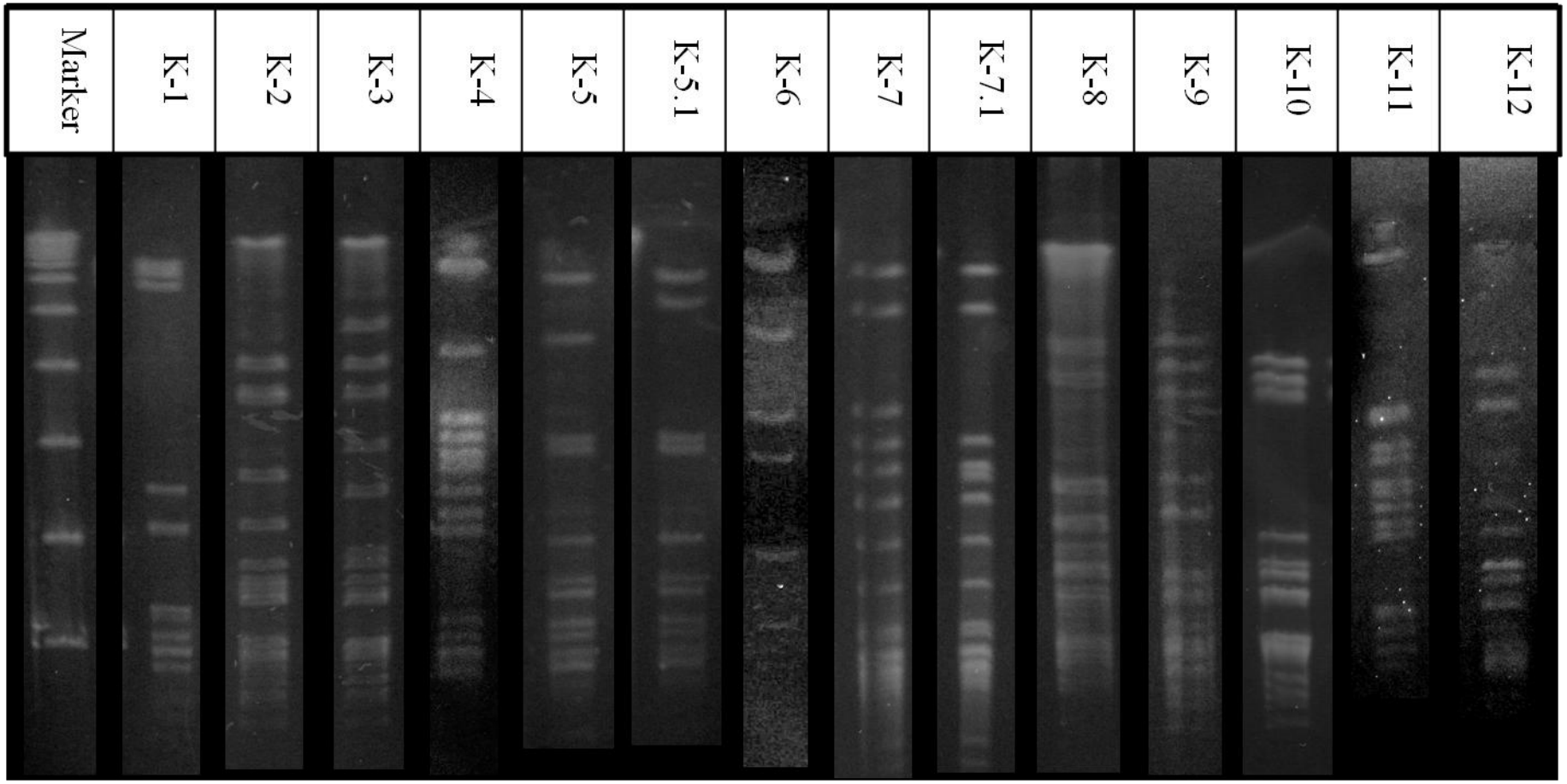
Biochemie: 30^{o}C Brutschrank

9.3 Molecular biological methods

9.3.1 DNA Preparation

- In case of *Campylobacter* DNA preparation, pure culture material was suspended in 1 ml distilled water, while the DNA of *E.coli* was prepared from an overnight culture in BHI broth (Brain Heart Infusion broth).
- The sample was centrifuged at 10,000 rpm for 5 min and the supernatant was removed.
- Then the pellet was resuspended in 200 µl distilled water and centrifuged at 10,000 rpm for 5 min.
- The supernatant was removed and resuspended in 500 µl distilled water.
- The bacterial suspension was boiled in a thermoblock at 100°C for 10 min and was left in an icebox at 0^{o}C for 10 min.
- After that it was centrifuged at 14,000 rpm for 1 min.
- The supernatant was taken in the new eppendorf and kept at -20°C.

9.3.2 Characteristics K1-K12 obtained from PFGE typing using enzyme Kpn-I



9.4 List of media, chemical reagents and equipment used in laboratory

9.4.1 Media and reagent

9.4.1.2 Media and reagents for microbiological analysis

Media	Article number	Company
Ammonium iron (III) sulphate solution, 1% • Ammonium iron(II) sulfate hexahydrate	 1.03792.0500	 Merck
Anaerocult® A	1.13829.0001	Merck
Barrit reagent (Alpha-naphtol solution) • 1-Naphthol • Ethanol	 1.06223.0050 9065.4	 Merck Roth
Blood Agar Base No.2	CM0271	Oxoid
Bolton broth selective supplement	SR0183	Oxoid
Bolton Selective Enrichment Broth	CM0983	Oxoid
Brain Heart Infusion broth (BHI)	48200	Serva
Brilliance Listeria agar base (OCLA)	CM1080	Oxoid
Brilliance Listeria selective supplement	SR0227	Oxoid
Brilliant green bile (2%) broth	CM0031	Oxoid
Brilliant-green Phenol-red Lactose Sucrose Agar (BPLS)	1.07232.0500	Merck
Brucella Broth	1.07228.0500/5007	Merck
Buffered Peptone Water	1.07228.0500/5007	Merck
Campylobacter Blood-Free Selective Agar Base (CCDA)	CM0739	Oxoid
Carbohydrate utilisation for *Listeria* • Proteose peptone • Meat extract • NaCl • Bromo cresol purple • Carbohydrate (only one of them for each test) - L-Rhamnose - D-Xylose - Arabinose - Mannitol	 0120-01 1.03979.0500 1.06404.0500 1.04736.0025 1.08692.1000 A9, 190-6 1.05982.0500	 Difco Merck Merck Feinchemie K.G.Sebnitz Merck Merck Sigma-Aldrich Merck
Carbohydrate utilisation for *Yersinia* • Bacto peptone • NaCl • Phenol red	 0118-01 1.06404.0500 31190	 Difco Merck Veb Laborchemie Apolda

Media	Article number	Company
• Carbohydrate (only one of them for each test)		
- Lysine	12042	Raanal
- Ornithine	15031	Raanal
- Rhamnose	1.04736.0025	Merck
- Saccharose	1.07687.025	Merck
- Trehalose	1.08216.0025	Merck
Catalase test	REF 55561	BioMérieux
CCDA Selective Supplement	SR0155	Oxoid
Cephalothin test discs	CT0010B	Oxoid
Citrate	1.02501.500	Merck
Columbia Agar (Base)	1.10455.0500	Merck
Cryobank™	CRYOG	Mast Diagnostics
Esculin agar (for *Yersinia*)		
• Meat extract	1.03979.0500	Merck
• Peptone from casein	1.07213.1000	Merck
• Esculin	1.00842.0025	Merck
• Ox bile	1.03756.0500	Merck
• Iron (III) citrate	1.03862.0500	Merck
• Agar	11398	Serva
Esculin broth (for *Listeria*)		
• Bacto peptone	0118-01	Difco
• NaCl	1.06404.0500	Merck
• Esculin	1.00842.0025	Merck
Fluorocult® ECD Agar	1.04038.0500	Merck
Gen Box ®microaer	REF 96125	BioMérieux
Greiss solution	1.09023.0500	Merck
Indol reaction		
• Peptone from Casein	1.07213.1000	Merck
• NaCl	1.06404.0500	Merck
• Tryptone	0123.01-1	Difco
Iron (III) chloride solution, 10%		
• Iron(III) chloride hexahydrate	1.03943.0250	Merck
Iron (III) citrate solution, 10%		
• Iron (III) citrate hydrate	1.03862.0500	Merck
Kligler's Iron Agar (KIA)	1.03013.0500	Merck
Kovac's reagent	1.09293.0100	Merck
Methylred solution		
• Methyl red sodium salt	1.06078.0025	Merck
Motility agar for *Listeria*		
• Peptone from meat	48620	Serva
• Peptone from casein	1.07213.1000	Merck
• Meat extract	1.03979.0500	Merck
• Yeast extract	2363.2	Carl Roth
• Glucose	1.04074.1000	Merck
• Agar	11398	Serva
Mueller-Hinton blood agar	CM337	Oxoid
Muller Kauffman Tetrathionate Broth with	1.05878.0500	Merck

Media	Article number	Company
Novobiocin (MKTTn)		
NaCl peptone solution		
• NaCl	1.06404.0500	Merck
• Peptone	1.07213.1000	Merck
Nalidixic acid test discs	CT0031B	Oxoid
Ninhydrin solution, 3.5% (w/v)		
• Ninhydrin 0.44 g	1.06762.0010	Merck
• Aceton 6.25 ml	8.2225.1000	Merck
• Isobutanol 6.25 g	1.00984.1000	Merck
Nitrate		
• Peptone from meat	48620	Serva
• NaCl	1.06404.0500	Merck
• Potassium nitrate	1.05063.0500	Merck
ONE broth Listeria base	CM1066	Oxoid
ONE broth-Listeria selective supplement	SR0234	Oxoid
Oxidase test	1.13300.0001	Merck
Oxidative-fermentative agar (glucose)	1.10282.0500	Merck
Paraffin	1.07160.1000	Merck
Potassium hydroxide solution, 40%		
• Potassium hydroxide pellet	1.050330.500	Merck
Pyrazinamidase agar		
• Tryptone Soya agar	CM131	Oxoid
• Tris-Maleate Buffer	T3128	Sigma-Aldrich
• Pyrazincarboxamid ($C_5H_5N_3O$)	8.21050.0025	Merck
RAMBACH® Agar	1.07500.0001	Merck
Rhamnose (for Yersinia)	1.04736.0025	Merck
Rhamnose for (Listeria)		
Saccharose (for Yersinia)	1.07687.250	Merck
Salicin	1076650025	Merck
Salmonella Enrichment Broth acc. to RAPPAPORT and VASSILIADIS (RVS Broth)	1.07700.0500	Merck
Sodium hippurate solution, 1.0% (w/v)		
• Na-Hippurat 2.5 g	8.20648.0025	Merck
• Phosphate buffer solution 250 ml		
(For 1000 ml PBS)		
- NaCl 8.5 g	1.06404.0500	Merck
- $Na_2HPO_4 \cdot 2H_2O$ 8.98 g	1.06404.0500	Merck
- $NaH_2PO_4 \cdot H_2O$ 2.71 g)	1.06346.0500	Merck
Standard I Nutrient Agar	1.07881.0500	Merck
Standard II Nutrient Agar	1.07883.0500	Merck
Triple sugar iron (TSI)	1.03915.500	Merck
Tween-Esterase medium		
• Peptone from meat	48620	Serva
• NaCl	1.06404.1000	Merck
• $CaCl_2$	1.02388.1000	Merck
• Agar	11398	Serva
• Tween 80 (Sorbitol mono-oleate)	9139.1	Roth

Media	Article number	Company
Urease for Listeria		
• medium base	1.08492.0500	Merck
• Urease	1.08487.0500	Merck
Urease and Tryptophan for *Yersinia*		Merck
• L-Tryptophan		Bundapest, Hungary
• Potassium dihydrogen phosphate	1.048729.025	Merck
• di-Potassium hydrogen phosphate	1.05104.1000	Merck
• NaCl	1.06404.1000	Merck
• Urease	1.08487.0500	Merck
• Phenolred		
• Iron (III) citrate hydrate	1.03862.0500	Merck
Violet Red Bile Dextrose (VRBD)	1.10275.050	Merck
Voges-Proskauer broth		
• Peptone from meat	48620	Serva
• Glucose	1.04074.1000	Merck
• NaCl	1.06404.1000	Merck
Yersinia Peptone, Sorbitol and Bile salt (PSB) broth		
• Bacto peptone	0118-01	Difco
• Sorbitol	1.07758.1000	Merck
• NaCl	1.06404.0500	Merck
• $Na_2HPO_4 \cdot 2H_2O$	1.06575.1000	Merck
• $NaH_2PO_4 \cdot H_2O$	1.06346.0500	Merck
• Ox bile	1.03756.0500	Merck
Yersinia selective agar base	CM0653	Oxoid
Yersinia Selective Agar Base and the selective supplement	SR0109	Oxoid
Yersinia Selective Enrichment Broth acc. to OSSMER	1.16701.0500	Merck
Zinc dust	1.08774.1000	Merck

9.4.1.2 Media and reagents for molecularbiological analysis

Reagent	Article number	Company
Agarose gel	2267.4	Carl Roth
AmpliTaq Gold DNA Polymerase	4311816	Roche
DNA-*Hind III* and Φx174 DNA-*Hae III* DNA marker	F-303 SD	Finnzymes

Reagent	Article number	Company
ESP solution		
- 0.5 EDTA	E 2.628-2	Sigma-Aldrich
- 1% N-Lauroyl-Sarcosine Sarkosyl	L-9150	Sigma-Aldrich
- 1mg/ml ProteinaseK, pH 9	03 115 801 001	Roche
Ethanol	9065.4	Carl Roth
Ethidium bromide	E-8751	Sigma-Aldrich
Faststart Taq Polymerase	04 738 420 001	Roche
*Kpn*I Buffer		
- 1M Tris-HCl pH7.5	4855.2	Carl Roth
- 1 M KCl	1.04936.0500	Merck
- 7M $MgCl_2$	1.05833.0250	Merck
- 2-Mercaptoethanol	8057400.250	Merck
KpnI Enzym	10 742 953 001	Roche
Liquid parafin	HP 50.3	Carl Roth
Loading buffer		
- 40 mg Na_2EDTA	39760	Serva
- 4 g Saccharose	50389	Sigma-Aldrich
- 3 g Bromophenolblue	1.08122.0025	Merck
MacFarland, 7		
9.3 ml 1%H_2SO_4	1.00731.1000	Merck
700 µl $BaCl_2$	1017190500	Merck
Megabase-Agarose	161-3108	BioRad
pBR328 DNA marker	X902.2	Carl Roth
Pett IV		
- 1M NaCl	3957.1	Carl Roth
- 10 mM Tris	5429.3	Carl Roth
- 10 mM EDTA pH8	39760	Serva
Pulse marker 50-1,000 kb	D-2416	Sigma-Aldrich
TBE buffer, 10x		
- 0.9 M Tris	5429.3	Carl Roth
- 0.9 M Boric acid	15165	Serva
- 0.025 M EDTA	39760	Serva
TE Puffer		
- 10 mM Tris	4855.2	Carl Roth
- 1 mM EDTA	39760	Serva
Ultra pure DNA grade Agarose	9012-36-6	Biorad

9.4.2 Equipment

9.4.2.1 Equipment for microbiological analysis

Equipment	Article number/model	Company
Balance	L2200S-D	Sartorius
Freezer (-30°C)	Premium	Liebherr
Incubator for 25°C		Melag
Incubator for 30°C	Kelvitron®t	Heraeus
Incubator for 30°C		Memmert
Incubator for 37°C	Kelvitron®t	Heraeus
Incubator for 42°C		Melag
Incubator for anaerobic condition	Vacutherm®	Heraeus
Incubator for microaerobic condition	Anaerobic workstation MK3	Don Whitley Scientific
Laboratory blender	Stomacher 400	Seward
Microscope	Dialux 20	Leitz-Wetzlar
Refrigerator	Standard 430	Kirsch
Refrigerator	Export	Bosch
Refrigerator	Profi line FKS2600	Liebherr
Thermometer/pHmeter	CG804	Schott

9 .4.2.1 Equipment for moleculabiological analysis

Equipment	Article number/model	Company
Autopipette for PCR, 0.5-10 µl	4910 000.018	Eppendorf
Autopipette for PCR, 100-1000 µl	4910 000.069	Eppendorf
Autopipette for PCR, 10-100 µl	4910 000.042	Eppendorf
Autopipette, 0.5-10 µl	4910 000.018	Eppendorf
Autopipette, 100-1000 µl	4910 000.069	Eppendorf
Autopipette, 10-100 µl	4910 000.042	Eppendorf
Balance	LP2200P	Sartorius
Balance	A200S	Sartorius
Blockthermostat	BT-200	Kleinfeld Labortechnik
Electrophoresis chamber		Roth
Electrophoresis chamber		Biotec Fischer
Gel document	Digital Imaging and Analysis System II (DIAS-II)	Serva
Laminar air flow	Flow laboratories HF48	Gelaire
Magnetic Stirrer	MR2002	Heidolph
Magnetic Stirrer	VMS-A	VWR
Microcentrifuge	5415R	Eppendorf

Equipment	Article number/model	Company
microtube with safetylock, 1.5 ml	0030 120.086	Eppendorf
microtube without safetylock, 1.5 ml	0030 125.150	Eppendorf
PCR tube, 0.2 ml	711080	Biozyme
PCR tube, 0.5 ml	711090	Biozyme
PFGE	Chef-DR®II	BioRad
Plug mold	1703706	BioRad
Power supply	Powerpac 300	BioRad
Power supply	Model 1000/500	BioRad
Refrigerater	4/-20°C	Bosch
Refrigerater	-20°C	AEG
Shaker	Certomat®HK	Sartorius
Spectrophotometer	Multiskan®Plus	Titertek
Stomacher	Laboratory Blender Stomacher 400	Seward
Thermocycler	Primus 96 plus	MWG-Biotech
Thermocycler	Trio-Thermoblock	Biometra
Thermomixer	5436	Eppendorf
Vortex	REAX2000	Heidolph
Waterbath		Kotterman

9.5 List of abbreviations

%	Percentage
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
A	Adenine
$BaCl_2$	Barium chloride
BHI	Brain Heart Infusion broth
bp	Base pair
BPLS	Brilliant-green Phenol-red Lactose Sucrose Agar
BT	Biotype
C	Cytosine
$CaCl_2$	Calcium chloride
CCDA	Campylobacter Blood-Free Selective Agar Base
CDC	Centers for disease control and prevention
CFR	Case Fatality Rate
CIN	Cefsulodin-Irgasan-novobiocin Agar Base (Yersinia selective agar base)
cm	Centimeter
CNS	Central Nervous System
CO_2	Carbon dioxide
DAEC	Diffuse-adhering *Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
ECD	*Escherichia coli* Direct Agar
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
EFTA	European Free Trade Association
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
et al.	et alii (and others)
ETEC	Enterotoxigenic *Escherichia coli*
EU	European Union
FoodNet	The Foodborne Diseases Active Surveillance Network
g	Gram
G	Guanine
h	Hour
H_2SO_4	Sulphuric acid
HCl	Hydrochloride
ISO	International Organization for Standardization
KCl	Potassium Chloride
Kp	Kilo base
$MgCl_2$	Magnesium Chloride

min	Minute
MKTTn	Muller Kauffman Tetrathionate Broth with Novobiocin
ml	Milliliter
mM	Millimolar
mm	Millimeter
mmol	Millimolarity
Na	Sodium
Na_2HPO_4	Disodium Hydrogen Phosphate
NaCl	Sodium Chloride
$NaH_2PO_4 \cdot H_2O$	Sodium Hydrogen Phosphate
O_2	Oxygen
^oC	Degree Celsius
OCLA	Brilliance Listeria Agar Base
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PSB	Yersinia Peptone, Sorbitol and Bile Salt Broth
RKI	Robert Koch Institute
rpm	Round per minute
RV Broth	Salmonella Enrichment Broth acc. to RAPPAPORT and VASSILIADIS
s	Second
STEC	Shiga-Toxins Producing *Escherichia coli*
T	Thymine
TSI	Triple Sugar Iron
USA	The United States of America
UV	Ultraviolet
V	Volt
VP	Voges-Proskauer Broth
VRBD	Violet Red Bile Dextrose
w/v	Weight by Volume
WHO	World Health Organization

9.6 List of tables

9.7 List of figures

10. Acknowledgments

I would like to acknowledge and extend my heartfelt gratitude to the Institute of Meat Technology and Hygiene (Free University, Berlin) for financial and technical supports, Free Univerity Berlin for granting my scholarship and sheep farms and the slaughterhouse for providing our samples and information.

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I am most grateful to my labmate, Iman Sharief. She is always my best friend during this time. We have been through the great time and some difficulties together. But no matter what happens, she always stands by me and makes our hard work much more fun. To express my feeling and thankful for her are more than I can say.

This thesis would not have been possible without the help and support of Lieselotte Bräutigam, Dorothea Jaeger, Herlinde Irsigler and Rosi Ludewig. I have learned some laboratory techniques from them, since I did not know any of German vocabulary. I am truly grateful for their understanding, kindness and patience.

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I would like to thank to my friends, Duangporn Pichpol and Anja Ludwig, for their friendship and encouragement. No matter what happen, happy or trouble, they have always been listening and understanding. Without them, my life in Berlin would not be so beautiful and easy.

I am heartily thankful my family, who unconditionally love me and always be there for me. Especially for my parents, who brought me up and guided me some useful attitude. I will never give up because of them.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 17.09.2010 Tongkorn Meeyam