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Isolation of lactic acid-related bacteria from the pig mucosal proximal gastrointestinal tract, including *Olsenella umbonata* sp. nov. and *Veillonella magna* sp. nov.

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Allen gewidmet, von denen ich lernte

—Günter Grass, *Beim Häuten der Zwiebel* (2006)

Contents

Abbreviations

16S	16Svedberg
adj.	adjective
Ala	alanine
ANI	Anaerobe Identification
AP	alkaline phosphatase
approx.	approximately
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BHG	brain-heart-glucose
BLAST	Basic Local Alignment Search Tool
bp	base pair
°C	degree Celsius
C4/C8/C14	4/8/14 carbon atoms
ca.	circa
Ca	calcium
$CaCl_2$	calcium chloride
CCUG	Culture Collection University of Gothenburg
CDC	Centers for Disease Control
CFU	colony-forming units
CIP	Collection de l'Institut Pasteur
CO_2	carbon dioxide
CoA	coenzyme A
$CuSO_4$	copper sulphate
d	day (time)
D-	D-configurated
DDBJ	DNA Data Bank of Japan
DIG	digoxigenin
DNA	deoxyribonucleic acid
DSM/DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
edn.	edition
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia (for example)
EMBL	European Molecular Biology Laboratory
et al.	*et alii*, *et aliae* (and others)
FAA	fastidious anaerobe agar
FCM-FISH	flow cytometry-fluorescent in situ hybridization

fem.	feminine (gender)
FEMS	Federation of European Microbiological Societies
$FeSO_4$	iron sulphate
Fig.	figure
g	gram
GC	gas chromatography
G+C-content	guanine and cytosine content
GI	gastrointestinal
JCM	Japan Collection of Microorganisms
Jr.	junior
h	hour (time)
HAP	hyper-ammonia-producing
HCl	hydrochloric acid
H_2O	water
H_2O_2	hydrogen peroxide
HPLC	high-pressure liquid chromatography
H_2S	hydrogen sulphide
i.e.	id est (that is)
IJSEM	International Journal of Systematic and Evolutionary Microbiology
INRA	Institut National de la Recherche Agronomique
kDa	kilodalton
K_2HPO_4	potassium monohydrogen phosphate
KH_2PO_4	potassium dihydrogen phosphate
l	litre
L.	Latin
L-	L-configurated
LAB	lactic acid-producing bacteria
LAMVAB	*Lactobacillus* Anaerobic MRS with Vancomycin and Bromocresol green
LARB	lactic acid-related bacteria
LDH	lactate dehydrogenase
LHP	*Lactobacillus*-hydrogen peroxide
LMG	Laboratorium voor Microbiologie en Microbiële Genetica
µg	microgram
µl	microlitre
µm	micrometer
m.	masculine (gender)
M	molar
max	maximal
ME	minimum-evolution

MEGA	Molecular Evolutionary Genetics Analysis
meq	milliequivalent
mg	milligram
$MgSO_4$	magnesium sulphate
min	minute (time) or minimal (OD)
ml	millilitre
mm	millimetre
mM	millimolar
mmol	millimol
$MnSO_4$	manganese sulphate
MP	maximum-parsimony
MRS	de Man-Rogosa-Sharpe
MRS-Van	MRS-Vancomycin
n	number (quantity)
n.	noun
N_2	dinitrogen (nitrogen gas)
***N*-**	nitrogen
NaCl	sodium chloride
NAD^+/NADH	nicotinamide adenine dinucleotide
Na_2HPO	sodium monohydrogen phosphate
NaH_2PO_4	sodium dihydrogen phosphate
NCBI	National Center for Biotechnology Information
NCIMB	National Collection of Industrial, food and Marine Bacteria
ng	nanogram
NJ	neighbour-joining
N.L.	Neo-Latin
nm	nanometre
nmol	nanomol
no.	number
nov.	nova (novel, new)
O_2	dioxygen (oxygen gas)
O-	oxygen-linked
OD/OD_{max}	optical density /maximal optical density
p-	para
p./pp.	page/pages
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGM	porcine gastric mucin
PGMM	porcine gastric mucus-mucosa

pH	pondus Hydrogenii; negative decadic logarithm of the molar concentration of dissolved hydronium ions
PHYLIP	PHYLogeny Inference Package
PJMM	porcine jejunal mucus-mucosa
pK_a	negative decadic logarithm of the acid dissociation constant K_a
PY	peptone-yeast extract
PYG	peptone-yeast extract-glucose
rDNA	ribosomal deoxyribonucleic acid
RDP	Ribosomal Database Project
Rogosa's SL	Rogosa's selective *Lactobacillus*
rRNA	ribosomal ribonucleic acid
s	second (time)
SB	Slanetz and Bartley
SCFA	short-chain fatty acids
SD	standard deviation
SEM	scanning electron microscopy
sp./spp.	species/species pluralis
subsp.	subspecies
T	type (type strain of species)
TES	Tris-EDTA-sucrose
TMB	tetramethylbenzidine
Tris	tris(hydroxymethyl)aminomethane
UBM	unsupportive basal medium
UK	United Kingdom
UPGMA	unweighted pair-group method with arithmetic mean
URAS	unreduced aerobically sterilised
UV	ultraviolet
vol.	volume
v/v	volume/volume
wk	week (time)
w/v	weight/volume

Chapter I

Scope and intention

The present thesis is about lactobacilli, other lactic acid-producing and lactic acid-fermenting bacteria [together termed herein 'lactic acid-related bacteria' (LARB)] from the pig mucosal proximal gastrointestinal (GI) tract. It describes the theoretical background for the design and application of two approaches for isolation of novel species of these groups of bacteria from the mucosae of the stomachs and jejuna of healthy domestic pigs. These approaches were developed based on what is invariably present in the otherwise often empty (or jejune) pig proximal gut: oxygen and mucins. As one approach was rewarding, the thesis also contains the descriptions of two novel LARB species: *Olsenella umbonata* and *Veillonella magna*.

I decided on this scope, because I had learned from my veterinary studies, and especially from the studies of animal nutrition, microbiology and pig diseases during my time of student research (Kraatz *et al.*, 2004; Taras *et al.*, 2005; Kraatz *et al.,* 2006; Taras *et al.*, 2006), that the intimate co-existence (symbiosis) of mucosa-associated lactic acid-producing bacteria (LAB) in the proximal GI tract is utterly relevant for both pig nutrition and health.

Lactobacilli and other acid-tolerant LAB are dominant members of the indigenous microbiota in the pig proximal GI tract due to their 'special foregut association' (Walter, 2008) with the non-glandular, stratified squamous epithelium of the stomach pars oesophagea (Savage, 1977; Tannock, 1992; Tannock, 2005; Bomba *et al.,* 2006; Krüger *et al.,* 2008). The stomach and small intestine of pigs are lactic acid habitats, in that lactic acid is the main microbial fermentation product found therein (Simon, 2008).

Being monogastric omnivores with a simple, quasi non-caecal GI system, pigs meet their energy and nutrient requirements predominantly by endogenous digestive processes in the stomach and proximal small intestine (Püschner & Simon, 1988; Simon, 2008). The glycocalyx of the jejunal brush-border presents the hotspot of terminal endogenous digestion and nutrient absorption (Moran, 1982; Püschner & Simon, 1988) as well as an interface of the mucosa-associated cell surface and mucus gel microhabitats (Lee, 1985; Laux *et al.,* 2005; Linden *et al.,* 2008), which are inhabited by the co-evolved, metabolically interactive and overall mutually beneficial indigenous microbiota (Tannock, 2005a; Ley *et al.,* 2008; Little *et al.,* 2008; Resta, 2009).

The pig GI tract may promote the overgrowth of potentially pathogenic members of the indigenous microbiota as well as growth of non-indigenous, pathogenic microorganisms. Hence the mucosal surfaces of the stomachs and small intestines of pigs are the target sites of infection for several bacterial enteropathogens (Cowart & Casteel, 2001; Waldmann & Plonait, 2001; Selbitz, 2002; Baele *et al.,* 2008). The indigenous gut microbiota normally provide 'colonisation resistance' (van der Waaij *et al.,* 1971) to the homeostatic GI tract ecosystem and thus inhibit enteropathogen infection (Lee, 1985; Chow & Lee, 2006; Little *et al.,* 2008; Stecher *et al.,* 2010).

The isolation and study of lactobacilli and other LAB in community with lactic acid-fermenting bacteria, i.e. of LARB communities, from the pig mucosal stomach and jejunum are a worthwhile scope, because these bacteria, more than other members of the proximal gut microbiota, exert profound and largely beneficial influences on both pig nutrition and health (Nousiainen *et al.,* 2004; Tannock, 2005; Richards *et al.,* 2005; Leser & Mølbak, 2009).

I was encouraged to isolate novel species of these groups of bacteria, as I knew this intention to be quite promising. It is estimated that the pig GI tract contains approximately ten times more microbial cells than are somatic mammalian cells in the pig body (Luckey, 1972; Savage, 1977; Leser & Mølbak, 2009) and that these microbial cells might represent 400 to even 10000 bacterial species (Leser *et al.,* 2002; Walter, 2008a). The majority of these species have not yet been cultivated, and their identity and characteristics are still unknown (Leser *et al.,* 2002; Richards *et al.,* 2005; Leser & Mølbak, 2009). Especially little is known about the bacterial composition at the mucosal surfaces of the pig small intestine (Simon *et al.,* 2004).

Culture-based isolation and characterisation of novel bacterial strains and species remain the 'gold standard' (Konstantinov *et al.,* 2004) in the analysis of the GI tract microbiota (Simon *et al.,* 2005; Camp *et al.,* 2009). These traditional methods still pave the way to unravel the open questions in gut microbial ecology, the fervent of which according to Little *et al.* (2008) and Tannock (2008) is: 'who is making up this community?' or 'whose habitat is this?'

I intended to find an answer to this question.

Outline

Chapter II presents a general introduction to the proximal GI tract ecosystem of pigs, with special focuses on the ecological relevance of lactobacilli and other LARB and on the role the mucosa-associated microhabitats have for host nutrition and the indigenous microbiota. Ultimately this chapter gives a preface to the methodological Chapters III and IV in that it outlines the theoretical background for the two newly developed culture-based approaches for isolation.

Chapter II has been written and submitted upon invitation to the 2010 edition of the Formatex Microbiology Book Series. It has been published:

Kraatz, M. (2010). Lactobacilli and other lactic acid-related bacteria in the mucosal proximal gastrointestinal tract of pigs: a review of ecology for two derivative approaches for isolation of novel species. In *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, vol. 1, The Formatex Microbiology Book Series, pp. 674-686. Edited by A. Mendéz-Vilas. Badajoz, Spain: Formatex Research Center.

Chapter III is about a first approach for isolation of novel species of lactobacilli. This approach was developed based on the presence of oxygen at the mucosae of the pig proximal GI tract and the production of hydrogen peroxide as a general response to oxygen of microaerotolerant lactobacilli. The chapter describes the validation and application of a *Lactobacillus*-specific culture medium, *Lactobacillus*-hydrogen peroxide, in combination with a screening catalase-benzidine dihydrochloride test using, in total, 48 strains of lactobacilli and other LARB and mucosal samples from the stomachs and jejuna of five pigs.

Chapter IV is about a second approach for isolation of novel species of LARB. It was developed based on the ecological function of host-derived mucins in the mucosa-associated microhabitats of the pig proximal GI tract. The chapter describes the application of PGM-plus and PGM, two habitat-simulating culture media containing porcine gastric mucin (PGM) as basal component, using mucosal samples from the stomach and jejunum of one pig. This mucin-based approach resulted in further polyphasic taxonomic studies of strains of *O. umbonata* sp. nov. and *V. magna* sp. nov.

Chapter V is about *O. umbonata*, a novel microaerotolerant LAB. The chapter contains a description of the species based on the identification and characterisation of four strains, one

of which was not from the study of Chapter IV but had originally been isolated from the rumen of a sheep at the Rowett Institute of Nutrition and Health in Aberdeen, Scotland (UK). Special emphasis was put on analysing functional attributes that permit life in the GI ecosystem and on delineating the ecological status of *O. umbonata*.

At the time of writing, Chapter V is accepted for publication:

Kraatz, M., Wallace, R. J. & Svensson, L. (in press). *Olsenella umbonata* sp. nov., a microaerotolerant anaerobic lactic acid bacterium from the sheep rumen and pig jejunum, and emended descriptions of *Olsenella*, *Olsenella uli* and *Olsenella profusa*. *International Journal of Systematic and Evolutionary Microbiology*. Published online April 30, 2010.

Chapter VI is about *V. magna*, a novel microaerotolerant lactic acid-fermenting bacterium. The description of the species was based on genotypic and phenotypic analyses of one strain from porcine mucosal jejunum (Chapter IV). Again, analysis of some ecologically relevant characteristics was incorporated into the taxonomic study.

Chapter VI chapter has been published:

Kraatz, M. & Taras, D. (2008). *Veillonella magna* sp. nov., isolated from the jejunal mucosa of a healthy pig, and emended description of *Veillonella ratti*. *International Journal of Systematic and Evolutionary Microbiology* 58, 2755-2761.

Chapter VII is the manuscript version of my poster presentation of the chief contents of Chapters IV to VI at the 7th Joint Symposium Rowett-INRA 2010 'Gut Microbiology: new Insights into Gut Microbial Ecosystems' in Aberdeen from June 23-25, 2010.

Chapter VIII gives a discussion about the major results and outcomes of the taxonomic studies of *O. umbonata* and *V. magna* (Chapters V and VI). The detection of the species is reviewed concerning the general composition of the pig proximal gut microbiota. Indigeneity as the presumed ecological status is discussed by looking at microaerotolerance, hydrogen peroxide accumulation and mucin utilisation as determinant phenotypic features. Furthermore, this chapter outlines the species' ecological niches in the pig jejunum and finally their relationship with the host.

Chapter IX is an appendix with a summary, an author's reference list, acknowledgments and a statement of originality.

Co-authors

This thesis comprises studies that were carried out in collaboration with other researchers.

Chapter V: Prof. John R. Wallace provided strain A2 of *O. umbonata* and conducted the analysis of cellular fatty acids of the olsenellae at the Rowett Institute of Nutrition and Health. He assisted in the interpretation of the results. Ph.D. Liselott Svensson performed DNA-DNA hybridizations with the *Olsenella* strains at the Culture Collection University of Gothenburg in Sweden. Prof. John R. Wallace and Ph.D. Liselott Svensson also contributed by proof-reading of the manuscript of Chapter V.

Chapter VI: Dr. David Taras guided my work until 2007/2008. He made significant contributions (euthanasia and gutting) to the sampling from pigs. He gave valuable advice on the isolation and the initial taxonomic analysis of the isolates from Chapter IV. Dr. David Taras also contributed by critical reading of the manuscript of Chapter VI.

The assistance of other colleagues is mentioned in the Acknowledgements sections of Chapters V and VI.

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Chapter II

Lactobacilli and other lactic acid-related bacteria in the mucosal proximal gastrointestinal tract of pigs: a review of ecology for two derivative approaches for isolation of novel species

Kraatz, M. (2010). Lactobacilli and other lactic acid-related bacteria in the mucosal proximal gastrointestinal tract of pigs: a review of ecology for two derivative approaches for isolation of novel species. In *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology,* vol. 1, *The Formatex Microbiology Book Series*, pp. 674-686. Edited by A. Mendéz-Vilas. Badajoz, Spain: Formatex Research Center.

This chapter reviews some ecological aspects of the gut microbiota of pigs, with particular emphasis on indigenous lactobacilli and other lactic acid-related bacteria (LARB) in the mucosal microhabitats of the stomach and jejunum. It depicts the way these bacteria make their living in the digestive and interdigestive proximal gastrointestinal tract and how oxygen as an omnipresent physicochemical environmental factor determines their mucosal niches. Overall, the chapter outlines the theoretical background of two new approaches for isolation of novel species of mucosa-associated lactobacilli and LARB.

1. Ecological aspects of the pig gut microbiota

1.1 The pig gut microecosystem and microbiota

The gastrointestinal (GI) tracts of pigs and other mammals collectively constitute a substantial microbial habitat in the Earth's biospheric ecosystem (Turroni *et al.,* 2008). Taken individually, the GI tract of a pig is itself a microbial ecosystem or microecosystem. In common with any macroscopic ecosystem, it presents a functionally stable unit consisting of inhabiting communities of interdependent, living (biotic) organisms and non-living (abiotic), physical and chemical environmental factors in the same given area (Savage, 1977; Savage, 1977a; Tannock, 1995; Wikipedia, 2010). The natural environment that hosts the inhabited area is a structural unit (organ) of a living, multicellular organism, itself contributing various biotic factors to the ecosystem.

The gut microbiota of pigs, as those of other mammals, consist of distinct proximo-distal assemblages of species populations (communities), which are mostly made up of by anaerobic Gram-stain-positive bacteria ('bacteriobiota'). The pig GI tract ecosystem also hosts members of the two other domains of cellular life: prokaryotic *Archaea* and microscopic, unicellular *Eukarya*, i.e. fungi and protoctists (Richards *et al.,* 2005; Tannock, 2005; Leser & Mølbak, 2009). Moreover, diverse non-cellular viral forms of life reside inside the gut. Some viral agents, the phages, can predate microbial cells and expand gut microbial diversity by introducing their reservoirs of genetic material (Camp *et al.,* 2009).

1.2 Pig + gut microbiota = microbial '**superorganism**'

The somatic cells of the pig's body and the sum of microbial cells inside its GI tract are thought of as a predominantly (approximately 90 %) microbial 'superorganism' (Luckey, 1972; Savage, 1977; Lederberg, 2000; Leser & Mølbak, 2009), presenting an entity in evolution (Ley *et al.,* 2008; Turroni *et al.,* 2008). According to recent estimates on the pig and human gut, the GI tract contains as much as 10^{18} bacterial cells of 400 to 10000 different species (Leser *et al.,* 2002; Wilson, 2005; Turroni *et al.,* 2008; Walter, 2008a), whereby only 30-40 species of the 'true' or 'core' microbiota comprise up to 99 % of the total community and provide major metabolic activities (Hooper *et al.,* 2002; Tannock, 2005). The gut microbiota can be collectively thought of as the host's largest, metabolically active entity, a veritable 'microbial organ' (Turroni *et al.,* 2008). More than other organs of the host body, this microbial organ is rapidly renewable (Mackie *et al.,* 1999), in that its genome ('microbiome') encodes an enormously diverse metabolic potential, hence permitting adaptation of the superorganism to changing autogenic or allogenic factors, such as food and nutrient availability (Zoetendal *et al.,* 2006; Camp *et al.,* 2009). It is likely, therefore, that feral pigs in their changeable state obtain more benefit from harbouring a gut microbiota than do domesticated pigs in modern pig production (Tannock, 1995).

1.3 Ecological status: autochthonous or indigenous bacteria

The GI tract is an open ecosystem in contact with the outer environment (Savage, 1977; Hammes & Hertel, 2006; Krüger *et al.,* 2008). Therefore, the ecological status of bacteria in a given gut habitat (ecological section) is *per se* uncertain.

Bacterial species may be allochthonous, i.e. just transients originating from another different gut habitat or an external habitat. Species that are normally associated with all individuals due to adaptive coevolution with a given host species (Oh *et al.,* 2010) are termed 'autochthonous' or 'indigenous' (Dubos *et al.,* 1965; Savage, 1977), meaning 'found in a place where they

were formed' (Walter, 2005) or 'native to a particular habitat' (Tannock, 1995). These terms are widely used as synonyms (Savage, 1977), whereby 'indigenous' is more correct (Tannock, 1995). Indigenous bacteria are native, in that they naturally colonise all neonate host species individuals. They are early or pioneer colonisers of a particular gut habitat due to initial acquisition prior to birth, during the birth process or from the immediate environment after birth (Mackie *et al.,* 1999; Jiménez *et al.,* 2008; Gancarčiková *et al.*, 2009). It is tempting to hypothesize that the acid-tolerant 'milk souring' lactic acid-producing bacteria (LAB) (Orla-Jensen, 1919) have coevolved with the guts of mammals that innately rely on lactation for the nutrition of neonates. Indigenous bacteria establish stable species populations of characteristic size in the 'climax' microbial communities of all normal, i.e. naturally living and healthy, adult host species individuals (Savage, 1977; Tannock, 2005a). Their timely permanent or resident presence is often due to an intimate association with the gut mucosal microhabitats (Savage, 1977; Chow & Lee, 2006). Being able to grow and reproduce under the given environmental conditions, indigenous members of the gut microbiota are impelled to make their living by creating a specific niche or 'metabolic site' in a microhabitat (Savage, 1977; Wilson, 2005). Consequently, indigenous bacteria are of inherent metabolic significance, i.e. they necessarily accomplish a demonstrable ecological function in the GI tract (Tannock, 2005a).

1.4 Pig-gut microbiota symbiosis

Under natural conditions, the pig's life is obligately dependent on symbiosis of its indigenous gut microbiota (Tannock, 2005a; Wilson, 2005; Leser & Mølbak, 2009). The term 'symbiosis' describes a permanent intimate coexistence of dissimilar organisms, namely the host macrosymbiont and the organismal sum of its microbial microsymbionts. Symbiotic host-microbiota interactions are viewed in terms of a continuum from protagonistic mutualism, unilaterally benign and otherwise neutral commensalism to antagonistic parasitism (Hooper & Gordon, 2001; Wilson, 2005; Little *et al.,* 2008; Camp *et al.,* 2009). As, in the GI tract, the ecological relationship of any individual microbial organism is intricate and condition-dependent (Little *et al.,* 2008), life in coexistence with gut microbiota is a delicate balance between health and disease (Richards *et al.,* 2005; Tannock, 2008).

The relationship between the pig host and its indigenous GI microbiota is generally considered to be mutually beneficial or mutualistic (Camp *et al.,* 2009; Resta, 2009). The indigenous gut microbiota are essential mutualists for pig health and survival, at least in the absence of a sterile environment (Richards *et al.,* 2005; Little *et al.,* 2008). The mutualistic relationship is relative to the GI section, insofar as it tends towards antagonism in the small intestine (due to competition for endogenously digestible nutrients) and towards protagonism

in the large intestine (due to cooperation by exploitation of endogenously non-digestible nutrients) (Krüger *et al.,* 2008). Taken individually, the vast majority of bacterial species in the GI tract would be considered as commensals (Tannock, 2005a). The term 'commensalism' literally means life 'at table together' (Hooper *et al.,* 2002). A commensal species benefits by partaking nutrients and exploiting a niche, without negatively or positively influencing its host (Tannock, 1995; Turroni *et al.,* 2008).

The symbiotic pig-gut microbiota relationship may be described as 'covert parasitism' (Tannock, 2005a), because the microbial communities naturally comprise some potential pathogens that normally, i.e. with homeostatic mechanisms acting to sustain the mutualistic symbiosis, live in harmony with the host (Savage, 1977a). However, once the homeostatic mechanisms are disrupted under certain circumstances, the potential pathogens may easily overgrow (Chow & Lee, 2006) and the indigenous microbiota be a source of endogenous or opportunistic infection and disease (Vaughan *et al.,* 2002; Wilson, 2005; Zoetendal *et al.,* 2006).

1.5 Ecological impacts of the indigenous gut microbiota

The indigenous gut microbiota considerably impact on an array of biochemical, physiological and immunological features of the host (Richards *et al.,* 2005; Tannock, 2005; Pai & Kang, 2008). Germfree animals show significant local (GI tract) and systemic (body metabolism) differences compared with their naturally raised counterparts (Norin & Midtvedt, 2006; Allaoui *et al.,* 2009). The GI phenotypes that are characteristic of conventionally colonised hosts in contrast to germfree hosts are collectively known as 'microflora associated characteristics' (Hooper *et al.,* 2002; Tannock, 2008). The ecological functions that are provided by the indigenous microbiota can be put into two groups: nutritional and protective functions (Richards *et al.,* 2005).

Growth promotion due to enhanced nutrient exploitation and energy harvest is the most prominent benefit from nutritional functions (Bomba *et al.,* 2006; Lebeer *et al.,* 2008; Kleerebezem *et al.,* 2010). Germfree animals require a higher dietary caloric intake than conventional animals (Norin & Midtvedt, 2006). The 'microbial balance index' (Vaahtovuo *et al.,* 2007) is significantly associated with growth and weight gain of pigs, indicating that some major bacterial populations of the gut microbiota are essential factors affecting animal productivity and well being.

The overall greatest benefit is 'colonisation resistance' due to diverse microbial protective functions. The concept of colonisation resistance (van der Waaij *et al.,* 1971) relies on the 'niche exclusion principle' of Hardin (Hardin, 1960). Colonisation resistance presents a first-line mechanism of host defence against infection or intoxication by allochthonous organisms, including enteric pathogens, and inappropriate overgrowth of potentially pathogenic indigenous opportunists (Lee, 1985; Richards *et al.,* 2005; Wilson, 2005; Chow & Lee, 2006). The resistance of climax microbial communities to structural change following perturbation is based on the functional redundancy of phylogenetically diverse species populations (Little *et al.,* 2008).

2. Indigenous lactobacilli and other LARB in the pig proximal GI tract

2.1 LAB in dairy and gut microecology

The concept of LAB is historically associated with food and feed manufacture (Axelsson, 2004). The onset of systematic scientific research on LAB was in the field of the early twentieth century dairy industry. Lactic souring and coagulation of milk were required bacterial abilities for large-scale production and preservation of dairy products (Barbés, 2008). Orla-Jensen, a prominent dairy bacteriologist of that time, defined a set of phenotypic core criteria of 'the true LAB': Gram-stain-positive, non-motile, non-spore-forming rods or cocci that ferment carbohydrates and higher alcohols to chiefly lactic acid (Orla-Jensen, 1919). Whereas this phenotypic definition is generally valid even today (Axelsson, 2004), the introduction of modern molecular genetic methods into bacterial taxonomy revealed that LAB do not form a 'great natural group' (Orla-Jensen, 1919), but are widely allocated on two disparate phylogenetic lineages of the Gram-stain-positive bacteria, namely the phyla *Firmicutes* and *Actinobacteria* (Schleifer & Ludwig, 1995). The traditional definition of LAB is phylogenetically restricted in that it generally excludes the *Actinobacteria* (Holzapfel & Wood, 1995; Walter, 2005). However, as pointed out by Axelsson in 2004 (Axelsson, 2004), it is actually only the Gram-positive cell wall characteristic that cannot be challenged as a criterion for LAB. Therefore, from the view of modern gut microecology, only a broad physiological definition of LAB as a group of 'Gram-stain-positive bacteria producing lactic acid in sole, principal or important amounts ($\geq$ 1 meq 100 ml^{-1} of culture) from the fermentation of sugars' is apt (Tannock, 1995; Inês *et al.,* 2008). In contrast to the traditional definition, this ecological definition allows for the production of considerable amounts of by-products or even a main end product other than lactic acid by phylogenetically diverse species populations (e.g. acetic acid by bifidobacteria). Lactic acid is a metabolic product from the fermentation of carbohydrates by many organisms in the mammalian intestine, including

clostridia, eubacteria and peptostreptococci (Konstantinov *et al.,* 2006). The affiliation of these organisms to the group of LAB is an open question of gut microecology.

2.2 LARB in gut microecology

Following the introduction of modern molecular methodologies, microbial ecologists have come to learn that the digestive tracts of mammals are ecosystems essentially different from a dairy fermenter (Tannock, 1999). Only the conditions in gnotobiotic animals (ex-germfree animals after controlled association with pure cultures) are somewhat comparable to those in the dairy. Naturally, the GI tract is an outside-open, multifactorial superorganism ecosystem comprising undefined, complex and self-regulating polymicrobial consortia with numerous interdependencies (Savage, 1977; Tannock, 2005a; Bomba *et al.,* 2006).

The GI tract is equivalent to a chemostat with steady state conditions (Lee, 1985; Tannock, 2008). The production of lactic acid by LAB normally does not lead to lactic acid accumulation and lactic acidosis of the gut. Resulting from a long-term reciprocal evolution of LAB with lactic acid-fermenting bacteria that have lost the glycolytic pathway (Wilson, 2005; Little *et al.,* 2008), complex food webs of lactic acid-related bacteria (LARB) present an intrinsic factor promoting gut ecosystem structural and functional stability (Chassard *et al.,* 2008). Food chains of LARB comprise a main example of 'niche construction' through synergistic nutritional interactions (syntrophism or interspecies cross-feeding) (Wilson, 2005; Walter, 2008a). The LAB belong to the 'core' or 'true' microbiota, and the lactic-acid related trophic chain is considered to be one of the principal metabolic pathways in mammalian gut ecosystems (Tannock, 1995; Tannock, 2005; Konstantinov *et al.,* 2006).

The Gram-stain-positive LAB can be grouped into the 'LAB *sensu stricto*' and the 'LAB *sensu lato*' (Inês *et al.,* 2008). Both groups are collectively termed 'lactic microbiota' (Walter, 2005) and, at the time of writing, comprise around 50 genera (Inês *et al.,* 2008).

2.2.1 LAB *sensu stricto* including *Lactobacillus*

The LAB *sensu stricto* are non-spore-forming members of the phylum *Firmicutes* and thus have a guanine and cytosine content (G+C-content) of genomic DNA of less than 53-55 % (Schleifer & Ludwig, 1995; Inês *et al.,* 2008). This low-G+C-content group of LAB comprises the historic 'milk-souring organisms' of Orla-Jensen (Orla-Jensen, 1919), with the core genera *Lactobacillus*, *Leuconostoc*, *Weissella*, *Pediococcus*, *Streptococcus* as well as *Lactococcus* and *Enterococcus* and, in the pig GI tract, also *Gemella* (Leser *et al.,* 2002;

Axelsson, 2004; Hammes & Hertel, 2006). The genus *Lactobacillus* historically comprises all rod-shaped LAB and with 167 species, at the time of writing, is still by far the largest genus (Euzéby, 1997). On the basis of two main carbohydrate fermentation pathways, the genus *Lactobacillus* is traditionally arranged into three physiological fermentation type-groups (obligate homofermenters, facultative and obligate heterofermenters) correlating with the three subgenera of Orla-Jensen (Orla-Jensen, 1919; Barbés, 2008). However, these groups correlate little with the phylogenetic subgroups, namely the *Lactobacillus delbrueckii* group, *L. reuteri* group and *L. salivarius* group, which were revealed by most recent taxonomic analysis of 16S rRNA gene sequences and probably represent different genera (Axelsson, 2004; Hammes & Hertel, 2009).

Due to the 'special foregut association' (Walter, 2008a), i.e. formation of true biofilms on the epithelial surfaces of the oesophagus and the pars oesophagea of the stomach (Tannock, 1992), many lactobacilli and other LAB *sensu stricto* belong to the indigenous bacteria in the pig GI tract (Vaughan *et al.,* 2002; Walter, 2005; Walter, 2008a). Lactobacilli are generally regarded as safe commensals with no distinct pathogenic potential for humans or animals (Hammes & Hertel, 2006; Barbés, 2008; Lebeer *et al.,* 2008; Hammes & Hertel, 2009). The group of LAB *sensu stricto* has a more dual nature. Especially some intestinal enterococci, in particular *Enterococcus faecalis*, can be opportunistic pathogens and implicated in disease (Axelsson, 2004). The fermentative properties of gut bacteria are in the focus when elucidating their ecological role for pig health (Niba *et al.,* 2009). The LAB are regarded the most beneficial part of the indigenous gut microbiota (Nousiainen *et al.,* 2004). The presence of lactobacilli and other LAB in the GI tract is generally considered to be advantageous to the pig host (Naito *et al.,* 1995), whereby an increased lactobacilli:enterococci ratio has been suggested to serve as an index of the health status (Collado & Sanz, 2007).

2.2.2 LAB *sensu lato* including *Bifidobacterium*, *Atopobium* and *Olsenella*

The LAB *sensu lato* are either spore-forming members of the phylum *Firmicutes* (e.g. bacilli, paenibacilli and sporolactobacilli) (Fritze & Claus, 1995) or members of the phylum *Actinobacteria* and thus have a G+C-content of more than 53-55 %. The genera *Bifidobacterium*, *Atopobium* and *Olsenella* constitute the high-G+C-content group of the LAB *sensu lato* (Inês *et al.,* 2008). Bifidobacteria such as '*Bacterium bifidum*' (Orla-Jensen, 1919) are historically considered to belong to the LAB (Axelsson, 2004). They possess a rather unique pathway of hexose fermentation, the so-called 'Bifidus pathway', resulting in acetic and lactic acids in a molar ratio of 3:2 (Tannock, 1992; Schleifer & Ludwig, 1995; Hammes & Hertel, 2009). The genera *Atopobium* and *Olsenella* are related closely to each other and only distantly to bifidobacteria and other *Bifidobacteriaceae* in that they form a 'bigeneric

branch' of the family *Coriobacteriaceae* (Kraatz *et al.,* in press). Both genera contain species that were reclassified from *Eubacterium*, *Lactobacillus* or *Streptococcus* (Collins & Wallbanks, 1992; Dewhirst *et al.,* 2001). It is likely that especially atopobia, olsenellae and other members of the high-G+C-content group of the LAB *sensu lato* are still underestimated inhabitants of the pig GI tract, from the results of both culture-based studies (due to unknown fastidious needs and relatively low tolerance to oxygen) and molecular genetic studies (due to the high G+C-content of DNA) (Farris & Olson, 2007; Frank *et al.,* 2008).

2.2.3 Functional redundancy of LAB in the mammalian GI tract

Most of the metabolically functional groups of microorganisms are present at rather similar levels in the guts of all healthy adult individuals (Chassard *et al.,* 2008). Hence, the overall functional metabolic profile ('microbial metabolome') is similar for the host species pig despite numerous individual species-level phylotypes of the predominant bacterial communities (Tannock, 2005; Turroni *et al.,* 2008; Camp *et al.,* 2009). The relatively sparsely inhabited sections of the proximal GI tract are 'lactic acid habitats', in that lactic acid is the predominant microbial fermentation product therein (Simon *et al.,* 2004; Goméz, 2006; Apajalahti *et al.,* 2009; Pieper *et al.,* 2008; Simon, 2008), whereas, in contrast to this, lactic acid is seldom detected as a major fermentation product of the rich communities of the large intestine or faeces (Tsukahara & Ushida, 2001; Duncan *et al.,* 2004). The concomitant presence of LAB *sensu stricto* and *sensu lato* in the GI tracts of pigs and other mammals is an important example of metabolic or functional redundancy of different phylogenetic lineages and, as such, an ecological basis for the intrinsic functional resilience and hence temporal-structural stability of climax microbial communities (Lee, 1985; Little *et al.,* 2008; Turroni *et al.,* 2008; Walter, 2008a).

2.2.4 Lactic acid-fermenting bacteria including *Veillonella*

Whereas the identity of the functionally specialised lactic acid-fermenting bacteria is largely unknown in the human gut, it is well established that, in the pig GI tract, species of the genera *Veillonella*, *Megasphaera* and *Selenomonas* are capable of converting lactic acid to largely propionic and acetic acids (Duncan *et al.,* 2004). According to Chassard and co-workers (Chassard *et al.,* 2008), the group of lactic acid-utilising bacteria is even greater in that it comprises, besides propionic acid-forming species, butyric acid-producing and sulphate-reducing bacteria. Notably, also the propionic- and butyric acid-forming bacteria are 'acidogenic', however, their fermentative end products have ecological impacts considerably different from those of lactic acid (Ouwehand & Vesterlund, 2004).

2.3 Colonisation of LARB in neonate pigs

In most neonate mammals, prior to or at the latest soon after birth, the gut microbiota are composed of enterobacteria but primarily of LAB (Savage, 1977). The pioneering LAB, mostly lactobacilli, streptococci and enterococci, colonise throughout the GI tract and predominantly in the stomach from the time the piglet first suckles the sow (Smith, 1965; Fuller *et al.,* 1978; Pedersen & Tannock, 1989; Tannock, 1992; Naito *et al.,* 1995; Mackie *et al.,* 1999; Bomba *et al.,* 2006; Goméz, 2006; Konstantinov *et al.,* 2006; Krüger *et al.,* 2008). The production of lactic acid by LAB is a major autogenic or intrinsic factor impacting on further successional colonisation. The presence of lactic acid leads to an early synergistic establishment of coexisting lactic acid-fermenting bacteria, including above all veillonellae and megasphaerae (Smith, 1965; Smith, 1965a; Kenworthy, 1973; McGillivery & Cranwell, 1992; Konstantinov *et al.,* 2006). Together, LAB and the functionally specialised lactic acid-fermenting bacteria constitute the pioneering group of LARB in the 'microbial ecosystem' (Thompson *et al.,* 2008) of neonate pigs.

2.4 LARB in adult pigs

The pioneer colonisers in neonate animals generally produce the offspring that eventually form the climax microbiota in the 'superorganism ecosystem' of adults (Savage, 1977; Thompson *et al.,* 2008). Hence, populations of LARB species acquired early in suckling piglets remain prevalent in adult pigs. Using rather simple culture-based methods, it had been recognised that the major components of the indigenous microbiota are Gram-stain-positive LAB, more precisely lactobacilli, streptococci and enterococci, throughout the different regions of the adult pig GI tract (Smith, 1965a; Kenworthy, 1973; Henriksson & Conway, 1996). More recently, the introduction of molecular genetic methods into gut bacteriology has confirmed the earlier findings (Leser *et al.,* 2002; Richards *et al.,* 2005). Lactobacilli and other LAB shed from the foregut epithelia inoculate digesta and may proliferate in the stomach, small intestine and the reminder of the gut (Smith, 1965; Fuller *et al.,* 1978; Pedersen & Tannock, 1989; Walter, 2005; Bomba *et al.,* 2006). LAB are among the predominant mucosa-associated bacteria in the overall GI tract of adult pigs (Tannock, 2005; Collado & Sanz, 2007). In concomitance with LAB, lactic acid-fermenting bacteria of the genus *Veillonella* are found regularly as indigenous inhabitants of all gut sections [reviewed by Kraatz & Taras (2008)].

2.5 LARB in the pig proximal GI tract

The indigenous microbiota are not distributed randomly throughout the GI tract, but instead are found at population levels and in species distributions that are characteristic of specific

sections (Mackie *et al.,* 1999). The pig proximal GI tract contains relatively low numbers of indigenous microbes due to the acid conditions in the stomach and the swift flow of contents in the proximal small intestine (Savage, 1977; Tannock, 2005). Unlike the bulk of gut microbes, LAB and especially lactobacilli are acid-tolerant (Tannock, 1992; Axelsson, 2004; Chow & Lee, 2006) and generally able to adhere to and colonise the mucosal surfaces (Goméz, 2006). Consequently, it has been stated by many authors that communities of LARB, containing mainly lactobacilli and streptococci, are ecologically relevant especially in the proximal GI tract of pigs (Alexander & Davies, 1963; Kovács *et al.,* 1972; Simon *et al.,* 2004a; Richards *et al.,* 2005; Bomba *et al.,* 2006; Hammes & Hertel, 2006; Maré *et al.,* 2006).

3. The mucosal proximal GI tract of pigs

3.1 Omnivores with a simple, quasi non-caecal GI system

The mode of nutrition and gut morphology type have a strong impact on the overall gut microbial community patterns of mammals (Ley *et al.,* 2008; Leser & Mølbak, 2009). Pigs and humans are omnivores possessing a continuous (straight-tube), simple GI system without a functional caecum (Moran, 1982; Püschner & Simon, 1988; Simon, 2008). In contrast to ruminants, pigs have first approach to their own food, the majority of which they digest by their own (endogenous) enzymes in the proximal GI tract and comparatively little by bacterial enzymes in the distal GI tract (Savage, 1977; Bomba *et al.,* 2006; Simon, 2008). Hence the function of the proximal gut of pigs is the endogenous hydrolytic degradation of macromolecular food constituents [proteins, carbohydrates (i.e. disaccharides lactose and saccharose as well as α-polysaccharides starch and glycogen), fats] into low-molecular mass, assimilable components (amino acids, monosaccharides, monoglycerids, glycerol, free fatty acids) and subsequent absorption of the components for systemic distribution (Wilson, 2005; Simon, 2008). Pigs may meet at most 10-30 % of their energy requirements through absorption of bacterial fermentation products (short-chain fatty acids) in the distal GI tract (Collinder *et al.,* 2003; Simon, 2008).

3.2 Anatomy and physiology of the pig proximal GI tract

The stomach and small intestine of pigs present the proximal parts of the barrel gut (König *et al.,* 1999) and encompass two of the three main digestive sections, with a total volume capacity of about each 30 % (Moran, 1982; Simon, 2008). The pig stomach is of the one-cavity and compound type (Püschner & Simon, 1988; König *et al.,* 1999; Simon, 2008). Two incompletely separated, functionally different compartments can be distinguished by two distinct types of inner lining (mucosa), namely the non-glandular forestomach around the

entry of the oesophagus into the hood-shaped diverticulum ventriculi (ca. 5 % of the inner surface area) and the secretory and essentially non-absorptive glandular stomach (ca. 95 %) (Henriksson *et al.,* 1995; Vollmerhaus & Roos, 1999; Pearson & Brownlee, 2005). The stomach of pigs is the site for food storage and, after the mouth, the second stage of mechanical and initial endogenous enzymatic digestion (Moran, 1982; Leser & Mølbak, 2009). The small intestine of pigs is comprised of the duodenum, the jejunum and the ileum from cranial to caudal end (König *et al.,* 1999). In the fully grown pig, it generally approximates 18 meters with almost 90 % as jejunum (Moran, 1982; Vollmerhaus & Roos, 1999). The jejunum is the site where the majority of endogenous digestion ensues and the overwhelming proportion of nutrient absorption (Moran, 1982; Chow & Lee, 2006). Remarkably, despite their physiological importance, only little is known about the bacterial colonisation of the pig stomach and jejunum (Simon *et al.,* 2004; Su *et al.,* 2008).

3.3 Mucosal microhabitats of the pig proximal GI tract

The three-layered GI tunicae mucosae are mucous membranes, because the innermost lining 'moist epithelia' are invariably coated with a layer of mucus (Liebich, 1999; Bacha & Bacha, 2000; Pearson & Brownlee, 2005). This mucus layer is a gel bilayer occurring in two distinct physical forms: an inner thin layer of stable, highly viscous mucus firmly adhering to the epithelial surface and an outer sloppy layer which is quite viscous but mixes with the luminal digesta (Wilson, 2005; Chow & Lee, 2006). The microvillous apical membrane of the intestinal epithelial cells (enterocytes) is covered by the so-called brush border glycocalyx, a microfilamentous web of cell-surface mucins (Bacha & Bacha, 2000; Linden *et al.,* 2008). The enterocyte glycocalyx presents a non-covalent interface, in that the cell-surface mucins interact with the secreted gel-forming mucin glycoproteins of the inner mucus gel layer, so helping it to remain associated with the epithelial surface (Wilson, 2005; Linden *et al.,* 2008). The pig proximal GI tract is characterised by its pronounced secretion of mucins due to exocrine cells in the extensive cardiac gland region of the glandular stomach and the mucous Brünner's glands in the submucosae of the duodenum and jejunum (Moran, 1982; König *et al.,* 1999; Liebich, 1999; Vollmerhaus & Roos, 1999; Bacha & Bacha, 2000; Breves *et al.,* 2000). The mucus gel bilayer serves as a protective lubricant and is part of the unspecific, innate host defence system (Pearson & Brownlee, 2005; Wilson, 2005). The mucosal epithelia and the interfaced mucus gel present a first-stage dynamic 'diffusion barrier' at which important exchange (absorptive and excretive) functions and ecological host-microbe interactions take place (Liebich, 1999; Pearson & Brownlee, 2005; Wilson, 2005).

3.4 Mucosa-associated bacteria in the pig proximal GI tract

To colonise the gut, bacterial population levels need to be stable in size over time, by doubling of cells at a rate that resists wash-out (Chow & Lee, 2006; Goméz, 2006). In pigs, only the distal ileum and the large intestine exhibit prolonged stasis allowing the luminal digesta to be colonised (Savage, 1977). In the pig stomach and jejunum, due to host defensive rapid peristaltic movements and great amounts of flushing endogenous fluids, the resident, indigenous microbiota are normally restricted to the mucosal microhabitats (Tannock, 1995; Hooper *et al.,* 2001; Ouwehand & Vesterlund, 2004; Simon *et al.,* 2005; Tannock, 2005; Wilson, 2005). Indigenous bacteria of these 'lotic' habitats are necessarily directly adhered to or indirectly associated with the mucosal surfaces (Pedersen & Tannock, 1989; Tannock, 1992; Tannock, 2005; Chow & Lee, 2006). The epithelial cell-surface and secreted gel-forming mucins are presumably the key factors in the mucosal association and persistence of the proximal gut microbiota (Nousiainen *et al.,* 2004; Camp *et al.,* 2009). As a pattern of spatial mucosal colonisation, the epithelial surface is a different microhabitat than the firmly adherent mucus layer and is colonised by distinct microbial communities (Lee, 1985; Wilson, 2005; Chow & Lee, 2006; Camp *et al.,* 2009). The specific binding of bacterial proteins (lectins) to defined O-linked oligosaccharides on mucin glycoproteins is an ecological basis for the host species-specific mucosal association of LAB and other indigenous bacteria (Savage, 1979; Tannock, 1990; Vandevoorde *et al.,* 1992; Wilson, 2005; Chow & Lee, 2006).

In pigs, the surfaces of the stomach and small intestine are densely populated with indigenous mucosa-associated bacteria (McGillivery & Cranwell, 1992; Bomba *et al.,* 2006; Collado & Sanz, 2007), including lactobacilli and other LAB *sensu stricto* (Dubos *et al.,* 1965; Tannock & Smith, 1970; Fuller *et al.,* 1978; Pedersen & Tannock, 1989; Walter, 2008a) that markedly interact with the structure of the GI mucosae (Nousiainen *et al.,* 2004). Due to their prolonged contact with the epithelial surfaces, mucosa-associated bacteria are potentially of great ecological significance and might exert beneficial, health-promoting actions on the pig host (Tannock, 1992; Richards *et al.,* 2005; Barbés, 2008).

3.5 Coaggregation, microcolonies and biofilms

Specific lectin-carbohydrate interactions are usually the basis for interbacterial adherence (coaggregation) (Ledder *et al.,* 2008). Coaggregation facilitates the formation of multicellular microcolonies on the GI epithelial surfaces (Wilson, 2005). Microcolonies are the predominant colonisation form in the human gut (Lebeer *et al.,* 2008). The enterocyte glycocalyx acts as a 'conditioning film' for the adherence of microbial aggregates and prevents intimate contact of bacteria with the host cell membranes (Lee, 1985; Wilson, 2005).

Microcolonies are often embedded in a matrix of bacterial extracellular polymeric substances, usually exopolysaccharides (Wilson, 2005; Lebeer *et al.,* 2008; Walter, 2008a; Kleerebezem *et al.,* 2010). In pigs, multispecies biofilms are formed from such growing microcolonies on the non-secretory, stratified squamous forestomach epithelium and on particulate matter in the large intestine (Wilson, 2005; Ledder *et al.,* 2008), rather than on the secretory columnar epithelia of the glandular stomach and small intestine (Wieler, 2007). The special foregut association in the pig stomach involves the formation of dense true biofilms of LARB, some of which adhere directly to the epithelial cells (Tannock, 1992, Tannock, 2005; Lebeer *et al.,* 2008; Tannock, 2008; Walter, 2008a; Leser & Mølbak, 2009).

3.6 LARB in the mucus gel layers

Presenting receptors for bacteria to adhere and colonise the mucosal GI tract, the complex mucin macromolecules of the mucus gel layers provide multiple ecological niches for indigenous microorganisms (Dubos *et al.,* 1965; Wang *et al.,* 2008). Lactobacilli and other LAB *sensu stricto* have a marked ability to persist in the lotic sections of the proximal GI tract by specific protein binding to mucins and other glycoproteins of mucus (Henriksson & Conway, 1996; Hammes & Hertel, 2006). Some intestinal lactobacilli exhibit cell surface polysaccharides, mostly exopolysaccharides, which resemble host mucins in the composition of the oligosaccharide side chains (Lebeer *et al.,* 2008; Kleerebezem *et al.,* 2010). Genes encoding for cell-surface proteins with specific mucin and mucus-binding domains (lectins) are broadly distributed among gut species of lactobacilli and other LAB *sensu stricto* (Azcarate-Peril *et al.,* 2008; Lebeer *et al.,* 2008; Kleerebezem *et al.,* 2010). Some of these cell surface adhesion determinants are constitutively present, while others (such as the mucus- and mucin-binding protein Mub of *L. reuteri*, *L. mucosae* and *L. acidophilus*) are induced by mucins (Jonsson *et al.,* 2001; Vaughan *et al.,* 2002). Colonisation of mucus is a key mode of direct antagonistic interference of indigenous LARB against allochthonous species including enteric pathogens. Firstly, LARB occupy mucin receptor sites (‘exploitative competition’ for sites of adhesion or ‘competitive exclusion’). Secondly, they produce, besides lactic and short-chain fatty acids, diverse antimicrobial compounds that are accumulated to higher concentrations in the matrix of the rigid inner mucus gel layer (‘interference competition’ or ‘amensalism’) (Nousiainen *et al.,* 2004; Ouwehand & Vesterlund, 2004; Richards *et al.,* 2005; Wilson, 2005; Barbés, 2008; Little *et al.,* 2008).

4. Digestive life of bacteria on exogenous nutrients

Lactobacilli and other gut LARB are chemo-organotrophic and typically auxotroph organisms with fastidious nutritional requirements (Schleifer & Ludwig, 1995; Axelsson, 2004). They are generally associated with habitats that are rich in easily assimilable substrates, such as amino acids, simple carbohydrates and vitamins (Axelsson, 2004; Walter, 2005). Nutritionally balanced and energy-rich, modern pig high performance feeds provide easily fermentable carbohydrates as a major nutrient supply for the indigenous communities in the proximal GI tract (Pieper *et al.,* 2008). The mucus gel layers in the stomach and jejunum of pigs present nutrient-rich habitats, in that food nutrient compounds are brought in contact with the epithelial surfaces by digestive peristalsis (Moran, 1982; Walter, 2008a).

4.1 Nutrients in the digestive stomach

Mainly proteins are digested in the stomach. The digestion is initiated by the gastric enzyme pepsin and, in suckling piglets, also by chymosin (rennin) and cathepsin. It results in the liberation of polypeptides (Moran, 1982; Simon, 2008). The low pH in the stomach lumen contributes to proteolysis by denaturation of feed proteins and also endogenous mucins. Pigs possess a salivary amylase (ptyalin) that is partially active on starch in the stomach. Therefore, some α-gluco-oligosaccharides, maltose, maltotriose and α-dextrines are present, especially in the non-glandular forestomach. Due to an acid (HCl) hydrolysis of β-glycosidic bonds of hemicelluloses (Püschner & Simon, 1988), further glucose-containing oligosaccharides occur. Gastric lipase and, in case of digesta backlog, also the lipase from pancreas hydrolyse fats (triglycerides) into some diglycerides, monoglycerids and fatty acids (Breves *et al.,* 2000; Simon, 2008).

4.2 Nutrients in the digestive jejunum

In the jejunum, endogenous digestion takes place in the lumen (luminal digestion) and in close contact with the mucosal epithelium (contact digestion) through sequential action of pancreatic enzymes and membrane-bound enzymes of the enterocyte brush border (Simon, 2008). Pancreatic enzymes include endo- and exopeptidases (trypsin, chymotrypsin, elastase, carboxypeptidases A und B), α-amylase, lipases (lipase, colipase, phospholipase A2, cholesterinesterase) and nucleases (ribo-, desoxyribonuclease). Epithelial enzymes of the jejunal brush border comprise peptidases with different specificity (tri-, di-, amino- and carboxypeptidases), oligo- and disaccharidases (glucoamylase, 1,6-α-glucosidase, maltase, maltotriase, isomaltase, fructosidase as well as lactase in suckling pigs), monoacylglyceridlipase and phosphodiesterase and many enzymes for the digestion of nucleic acids (Breves *et al.,* 2000; Simon, 2008). The pancreatic peptidases and most of the lipases

need to be enzymatically activated at the jejunal brush border and, therefore, are active predominantly inside the adherent mucus gel layer (Püschner & Simon, 1988). The membrane-bound enzymes are active only on such nutrient compounds that are already small enough to diffuse through the mucus gel layer towards the enterocyte brush border (Püschner & Simon, 1988). Forming a loose, filamentous barrier between the epithelial cell surface and the inner mucus gel layer, the jejunal brush border glycocalyx separates particulates from solubles and presents the hot spot of terminal digestion and nutrient absorption (Moran, 1982). Hence, during digestive periods, the mucosa-associated bacteria have direct access to great amounts of readily absorbable substrates, namely simple carbohydrates (glucose, fructose, galactose, mannose), amino acids, di- and tripeptides, monoglycerids, fatty acids and glycerol as well as the different components of nucleic acids (Breves *et al.,* 2000).

4.3 Bacterial impact on terminal digestion and absorption

The indigenous bacteria in the stomach and small intestine of pigs significantly impact on nutrient conversions (Simon *et al.,* 2004a). Members of the mucosal gut microbiota affect intestinal epithelial physiology, including the activities of brush-border enzymes (Resta, 2009). They modify the epithelial expression of several host genes involved in the processing, absorption and metabolism of carbohydrates, lipids and micronutrients (Wilson, 2005; Leser & Mølbak, 2009). *In vitro* and in mice, lactobacilli and other indigenous bacteria promote energy harvest from the gut by genomic or rapid non-genomic upregulation of, *inter alia*, the epithelial sodium-dependent glucose transporter, pancreatic lipase-related protein and colipase (Hooper *et al.*, 2001; Hooper *et al.,* 2002; Bäckhed *et al.,* 2004; Kleerebezem *et al.,* 2010; Rooj *et al.,* 2010). Microbial metabolism in the pig mucosal proximal GI tract is an important factor on host systemic metabolism and energy storage (Bäckhed *et al.,* 2004; Leser & Mølbak, 2009; Bäckhed & Crawford, 2010).

5. Interdigestive life of bacteria on endogenous mucins

In modern pig production, restricted feeding regimens with a low frequency of highly digestible feeds bring about prolonged interdigestive periods considerably influencing bacterial community patterns in the proximal GI tract (Smith, 1965a; Moran, 1982; Tannock, 1992; Rodehutscord, 2008). Following digestion, the proximal GI tract is purified from residual nutrients by interdigestive propulsive activities (Breves *et al.,* 2000). Prolonged interdigestive periods (fasting) cause a marked reduction but not complete decolonisation of LAB and other indigenous bacteria (Smith, 1965a; Morishita & Ogata, 1970; Kovács *et al.,* 1972). Rapid reconstitution of community patterns is assured by the mucosa-associated

populations (Vandevoorde *et al.,* 1992) that rely on mucin glycoproteins as endogenous continuously available nutrients (Hooper *et al.,* 2002; Azcarate-Peril *et al.,* 2008; Chassard *et al.,* 2008; Hammes & Hertel, 2006). Mucins are the main macromolecular structural constituents of the aqueous mucus gel bilayers (Wilson, 2005). A great number of indigenous bacteria have coevolved the capability of utilising mucins as alternative sources of carbon, nitrogen and energy (Wilson, 2005; Walker *et al.,* 2006; Chassard *et al.,* 2008). During interdigestive periods, i.e. in the absence of exogenous carbohydrate sources, some bacteria show adaptive expression of genes for a number of enzymes for breaking down and utilising the mucin oligosaccharide side chains (Hooper *et al.,* 2002; Chow & Lee, 2006; Zoetendal *et al.,* 2006). Mucins consist to 70-85 % of carbohydrates, mainly repeating disaccharide units of galactose and *N*-acetylglucosamine, which present a major endogenous source for bacterial fermentation to lactic and short-chain fatty acids (Bradshaw *et al.,* 1994; Collinder *et al.,* 2003; Wilson, 2005).

5.1 Ecological stabiliser function of mucins

Degradation of mucins is not accomplishable only by host-derived proteases but needs the specific glycosidic actions of communities of cooperative bacteria linked in the syntrophic webs of microcolonies or biofilms (Hoskins, 1992; Ledder *et al.,* 2008; Little *et al.,* 2008; Turroni *et al.,* 2008). Providing continuously present metabolic sites (niches) for diverse bacterial communities, mucins accomplish an ecologically outstanding stabiliser function in the interdigestive proximal GI tract (Norin & Midtvedt, 2006; Turroni *et al.,* 2008). More diverse bacterial communities that occupy all niches in an ecosystem generally exhibit greater structural and functional robustness (Lee, 1985; Chow & Lee, 2006; Little *et al.,* 2008). Mucinolytic capabilities of mucosa-associated bacteria are very likely more relevant for maintaining a healthy ecosystem in the proximal GI tract of domestic pigs than of feral pigs. Selection for mucinolytic populations of LARB presumably takes places already in suckling piglets due to mucin-like oligosaccharides and glycoproteins in the sow's milk (Mackie *et al.,* 1999; Turroni *et al.,* 2008).

6. Adverse host-derived environmental factors of mucosal GI niches

In order for an indigenous organism to establish at a particular site, the environment of that site must be able to satisfy the organism's nutritional and physicochemical requirements, and the organism must be able to make a living at the site (Wilson, 2005). It is a sign of adaptive coevolution that at sites, like the pig proximal GI tract, where the host extracts many of the nutrients from food and where microorganisms are likely to compete with intestinal functions,

colonisation is governed by host-derived selecting factors (Lee, 1985; Wilson, 2005). Thus the mucosal niches of the pig stomach and small intestine are determined by a range of adverse mechanical, biological and physicochemical environmental factors (Wilson, 2005). Autochthony or indigeneity is based on the presence of genes for factors that allow bacteria to overcome adverse environmental conditions (Walter, 2008a). The formation of microcolonies and biofilms is an important microbial strategy for survival and is associated with longer persistence of bacteria in the GI tract (Crippen *et al.,* 2008; Fakhry *et al.,* 2009).

Adverse mechanical factors include removal forces due to digestive and interdigestive gut motor functions and propulsive activities (peristalsis) (Savage, 1977; Lee, 1985; Tannock, 1995, Breves *et al.,* 2000), villous motility of the small intestinal mucosa (Savage, 1979) and shedding and renewal (turn over) of the epithelial cell surfaces (Moran, 1982; Pearson & Brownlee, 2005). Examples of biological determinants are the diverse antibacterial compounds that are secreted by the mucosal cells or cells of the accessory digestive organs pancreas and liver/gallbladder (Liebich, 1999; Leser & Mølbak, 2009) as well as different immune responses of the gut-associated lymphoid tissue (Wilson, 2005; Gaskins *et al.,* 2008). Important physicochemical environmental factors are the pH as well as oxygen and redox potential (Wilson, 2005).

6.1 Oxygen as an omnipresent physicochemical factor

Oxygen molecules generally pass from the blood through the gut epithelia into the mucosal microhabitats (Savage, 1977). The stomach and small intestine of pigs are highly vascularised organs, and, therefore, close to the mucosa the oxygen contents are relatively high (Hammes & Hertel, 2006). In the stomach, the secretory mucosa of the oxyntic gland region is rich in alveolate webs of blood capillaries (Liebich, 1999) in order to sustain the energy-consuming secretion of HCl (Moran, 1982). Besides, small amounts of oxygen in air are regularly swallowed into the stomach by the intake of food or water (Wilson, 2005). Like the stomach of humans, the pig stomach is an essentially aerobic environment, exhibiting an oxygen content at the luminal mucosal surface of about 29 % of the oxygen content of air (Wilson, 2005). The absorptive mucosa of the jejunum exhibits a very tight microvascular epithelial network (Moran, 1982; Liebich, 1999; Stappenbeck *et al.,* 2002; Ogawa & Binion, 2005; Gancarčiková *et al.*, 2009). The oxygen content at the luminal surface of the small intestinal mucosa corresponds to about 22 % of the oxygen content of air (Wilson, 2005).

Because of its strong oxidising ability, the presence of oxygen exerts a severe effect on the redox potential in the environment (Wilson, 2005). Indigenous lactobacilli and other bacteria

encounter redox stress conditions associated with the oxygen gradients that are steep at the mucosal surfaces (Kleerebezem *et al.,* 2010). The oxygen content generally declines towards the gut lumen due to reduction by oxygen-tolerant microorganisms (Wilson, 2005). The presence of oxygen gradients at the mucosae of the proximal GI tract coincides with the establishment of complex microbial communities. The bacterial communities of the pig stomach and small intestine contain aerobes, facultative anaerobes and, as the typical LAB, aerotolerant to microaerotolerant (moderately obligate) anaerobes (Axelsson, 2004; Winn *et al.,* 2005; Krüger *et al.,* 2008).

7. Approaches for isolation of novel mucosa-associated lactobacilli and other LARB

Cultivation and other culture-based methods present the traditional techniques used to answer the prior question in gut microbial ecology, namely 'who is making up this community?' or 'whose habitat is this?' (Richards *et al.,* 2005; Little *et al.,* 2008; Tannock, 2008). They remain the 'gold standard' for isolating strains of novel species for representative phenotypic, genetic and molecular characterisation (Konstantinov *et al.,* 2004; Simon *et al.,* 2005; Camp *et al.,* 2009). The predominant part of the pig GI tract bacteriobiota has not yet been recovered in pure culture (Leser *et al.,* 2002; Richards *et al.,* 2005; Leser & Mølbak, 2009).

7.1 Specific isolation of haem-independent catalase-positive lactobacilli

The pig proximal GI tract harbours a major proportion of presumably unknown *Lactobacillus* species (Simon *et al.,* 2005a). Detection of isolates of novel species is likely to succeed best using alternative specific culture media and specifically adjusted conditions (Hammes & Hertel, 2009; Vera *et al.,* 2009). At the mucosae of the pig stomach and jejunum, the continuous presence of oxygen is likely to be a crucial factor in the selection for oxygen-detoxifying, microaerotolerant bacteria. The ability to generate hydrogen peroxide from oxygen is widespread among GI mucosal lactobacilli (Annuk *et al.,* 2003), however, lactobacilli usually do not possess catalases and are poorly equipped with other antioxidative enzymes for the degradation of accumulating hydrogen peroxide (Rochat *et al.,* 2006; Hammes & Hertel, 2009). Therefore, the one-step direct specific isolation and qualitative analysis of hydrogen peroxide-accumulation of microaerobically grown lactobacilli in combination with a screening catalase-benzidine dihydrochloride test (Deibel & Evans, 1960) for haem-independent manganese catalase of hydrogen peroxide-negative isolates present a promising approach in the detection of strains of novel species.

7.2 Isolation of complex communities of mucin-utilising LARB

The gut mucosal surfaces are very complex habitats which are hard to reproduce *in vitro* (Simon *et al.,* 2004). Mucosa-associated bacteria often take more time to grow on conventional media and, therefore, require more advanced, innovative cultural approaches (Simon *et al.,* 2004a). More advanced culture techniques incorporate various nutrients and abiotic conditions that closely mimic the environment from which the bacteria were isolated (Little *et al.,* 2008). Providing multiple nutritional niches, commercial porcine gastric mucin is a suitable basal component in a habitat-simulating approach for enrichment isolation of naturally complex and diverse mucosa-associated communities of LARB. A porcine gastric mucin medium with very low concentrations (0.001 %) of peptone, yeast extract and glucose imitates the interdigestive mucosal microhabitats of the pig proximal GI tract. Such a medium was successfully applied in the isolation of strains of two novel LARB species, namely *Olsenella umbonata* and *Veillonella magna* (Kraatz & Taras, 2008; Kraatz *et al.,* in press).

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Chapter III

LHP: a new medium for the specific direct isolation of lactobacilli and screening for strains of novel species with haem-independent manganese catalase activity on hydrogen peroxide

Introduction

Hydrogen peroxide is generated by almost all aerobically or microaerobically growing bacteria (Juven & Pierson, 1996). NADH:H_2O_2 oxidase and other non-haem flavoprotein oxidases for the production of hydrogen peroxide or water from traces of molecular oxygen are a universal property of the lactic acid-producing bacteria (LAB) *sensu stricto* (Condon, 1987; Sakamoto & Komagata, 1996). Production of hydrogen peroxide, first termed 'lactobacillin' by Wheater *et al.* (1952), is a widespread feature of gastrointestinal (GI) mucosal lactobacilli (Annuk *et al.,* 2003) and presents a well-recognised mechanism of antimicrobial and potentially probiotic action of these bacteria and other LAB *sensu stricto* (Nousiainen *et al.,* 2004; Hammes & Hertel, 2006).

The most important scavenger of hydrogen peroxide in the LAB *sensu stricto* is a non-haem flavoprotein NADH peroxidase (Condon, 1987; Brioukhanov & Netrusov, 2007). The haem-dependent true catalase, which also degrades hydrogen peroxide, is ubiquitously present in aerobic and facultatively anaerobic bacteria containing a functional cytochrome system (Percy, 1984; Schmitz *et al.,* 2006) and is generally missing in lactobacilli and other LAB *sensu stricto* when cultured in the absence of haem (Schleifer & Ludwig, 1995; Axelsson, 2004). However, a second type of catalatic activity has been observed in these bacteria (Felton *et al.,* 1953; Dacre & Sharpe, 1956) and identified as originating from a haem-independent 'pseudocatalase' (Delwiche, 1959; Johnston & Delwiche, 1962; Whittenbury, 1964) with manganese as the redox cofactor (Kono & Fridovich, 1983; Barynin *et al.,* 2001). The occurrence of this haem-independent manganese catalase is less frequent compared with the true catalase in the presence of a source of preformed haem, e.g. haemin or haematin, but has been described for some strains of lactobacilli, enterococci, pediococci and leuconostocs (Johnston & Delwiche, 1962; Whittenbury, 1964; Johnston & Delwiche, 1965; Engesser & Hammes, 1994). Among the lactobacilli, only two species, namely *Lactobacillus plantarum* subsp. *plantarum* and *L. mali*, exhibited positive strains to date (Carr & Davies, 1970; Engesser & Hammes, 1994; Bringel *et al.,* 2005; Hammes & Hertel, 2009).

Overall, lactobacilli and other LAB *sensu stricto* are generally relatively poorly equipped with antioxidative enzymes that effectively detoxify hydrogen peroxide (Rochat *et al.,* 2006). Hydrogen peroxide therefore usually accumulates and causes growth arrest in microaerobic cultures of these bacteria (van de Guchte *et al.,* 2001). In many studies (Whittenbury, 1960; Kono & Fridovich, 1983a; Wolf *et al.,* 1991; Hertel *et al.,* 1998), it has been observed that active catalases prevent accumulation of hydrogen peroxide in cultures of lactobacilli and other LAB *sensu stricto*.

I hypothesised that analysis of manganese catalase activity of hydrogen peroxide-negative *Lactobacillus* isolates presents an effective screening method for the detection of strains of novel species. Colony isolates from a haem-free and highly lactobacilli-specific medium that do not accumulate hydrogen peroxide under microaerobic culture conditions are more likely to possess a manganese catalase and hence represent a novel *Lactobacillus* species than hydrogen peroxide accumulating and catalase negative-isolates. A simple screening method for strains of potentially novel *Lactobacillus* species is expedient, since lactobacilli are numerous at the mucosal surfaces of the pig proximal GI tract (Tannock, 2005).

Most of the sensitive methods for detecting hydrogen peroxide use peroxidases, especially horseradish peroxidase, and a number of plating media based on horseradish peroxidase and a chromogenic substrate that becomes visually oxidised by a complex of peroxidase with hydrogen peroxide have been recommended for the analysis of hydrogen peroxide-producing bacteria (Juven & Pierson, 1996). 3,3´,5,5´-tetramethylbenzidine (TMB) has been introduced as a chromogenic substrate by Eschenbach *et al.* (1989) in their survey of human lactobacilli and vaginosis. Generally applicable in studies with the LAB *sensu stricto* (Juárez Tomás *et al.,* 2004), TMB is nowadays commonly used for the qualitative analysis of hydrogen peroxide production by lactobacilli from various sources (Felten *et al.,* 1999; Mota *et al.,* 2006; Otero & Nader-Macías, 2006; Pascual *et al.,* 2006). Hitherto, *Brucella* agar (in TMB medium; Eschenbach *et al.*, 1989), an enriched and lactobacilli elective version of *Brucella* agar (in TMB-plus medium; Rabe & Hillier, 2003; Mota *et al.,* 2006) and de Man-Rogosa-Sharpe (MRS) agar (Felten *et al.,* 1999; Juárez Tomás *et al.,* 2004; Otero & Nader-Macías, 2006; Pascual *et al.,* 2006) have been used as basal media for lactobacilli in combination with horseradish peroxidase and TMB.

However, according to the results of Rabe & Hillier (2003) TMB, TMB-plus and MRS medium are unsuitable for analysis of hydrogen peroxide due to the ingredient substances haemin, blood serum or meat extract (all containing haem), Tween 80 and citrate. Exogenous

haem compounds may be incorporated into the apoenzyme of the true catalase (Abriouel *et al.,* 2004), or they themselves destroy hydrogen peroxide (Jones *et al.,* 1964). Citrate forms chelate complexes with lactobacilli elective magnesium and manganese ions (MacLeod & Snell, 1947). Thus citrate and also Tween 80 were found to cause inhibition of the growth of some hydrogen peroxide-negative lactobacilli and/or lower production of hydrogen peroxide (Rabe & Hillier, 2003). Besides this, neither of these media is truly selective for lactobacilli from a complex and partially less fastidious microbiota, such as the GI microbiota of humans, pigs and other mammals (Carr *et al.,* 2002; Hammes & Hertel, 2006). Therefore, using mucosal samples from the pig proximal GI tract, it seemed necessary for me to create a new medium for the purpose of direct specific isolation and qualitative analysis of hydrogen peroxide accumulation of lactobacilli.

The manganese catalase of lactobacilli can be differentiated from true catalase by the benzidine dihydrochloride test of Deibel & Evans (1960). This test is specific for iron-porphyrin compounds of the haem prosthetic groups of haemoproteins and therefore detects respiratory cytochromes as well as true catalase, nitrate and nitrite reductase (Bascomb, 1987; Bascomb & Manafi, 1998). In the presence of iron-porphyrin compounds and hydrogen peroxide, benzidine forms quinoidic bonds that impart a blue colour to the reaction mixture (Deibel & Evans, 1960). Accordingly, a positive catalase reaction (effervescence upon addition of hydrogen peroxide to colony material of bacteria) and a subsequent negative benzidine reaction (no blue colour reaction upon addition of benzidine dihydrochloride) are indicative of a non-haem manganese catalase. Lactobacilli are generally unable to synthesise porphyrins and exhibit functional cytochromes, haem-dependent true catalase and nitrite reductase only due to an exogenous source of preformed haem and/or menaquinone (Wolf & Hammes, 1988; Axelsson, 2004; Brooijmans *et al.,* 2009; Hammes & Hertel, 2009). Therefore false identification of a catalase as true catalase can be precluded by using an appropriate culture medium that is free of haem.

The objectives of the present study on the microbiota of the pig mucosal stomach and jejunum were to: *i*) specifically isolate lactobacilli; *ii*) assess the rate of hydrogen peroxide accumulation of the isolates; and *iii*) detect strains of potentially novel *Lactobacillus* species by screening of hydrogen peroxide-negative colony isolates for manganese catalase activity using a simple catalase-benzidine dihydrochloride test.

Materials and methods

Pigs

Cross-bred (Euroc×Piétrain) domestic pigs (*Sus scrofa domestica*) (*n*=5) were used. The pigs were born and reared at the Institute of Animal Nutrition during the approved scientific study 'Integrative analysis of the modes of action of probiotics in pigs' (German Research Foundation research group FOR 438). They were weaned at four weeks of age. Four pigs were from a control group that had not received any probiotic feed, and one pig was from a probiotic group that had been fed the probiotic strain *Enterococcus faecium* NCIMB 10415 in form of the feed additive Cylactin LBC ME10 (Cernivet, Cerbios-Pharma SA). The pigs were euthanized at 62 (*n*=1; from the control group) or 31-32 (*n*=4) days of age. The 62-day-old pig ingested approx. 1.34-3.35 g chromium oxide (Cr_2O_3) per day in the course of a digestibility study for six days ante mortem (data from D. Taras, unpublished results). It was also utilised for the isolation of mucosa-associated lactic acid-related bacteria (LARB) applying PGM-plus and PGM, two newly designed mucin-based culture media (Chapter IV). All pigs were clinically healthy at the day of sampling.

Gutting and sampling

The pigs were anaesthetised with ketamin and azaperon and then euthanized by means of an overdose of sodium pentobarbital. Immediately after euthanasia, the abdominal cavity was opened by midline incision. The stomach and proximal jejunum were clamped, removed and the contents were stripped out in the proximodistal direction. Ingesta and luminal bacteria were washed off using the following two-step protocol: The stomach and proximal jejunum were placed in separate Schott flasks containing 500 ml of sterile 0.9 % NaCl solution, and the flasks were vigorously shaken by hand. After removal from the flasks, the organs were longitudinally opened, and the oesophageal, non-glandular part of the stomach was separated from the glandular part. The washing was repeated using a fresh flask for each of the three GI sections. The tunicae mucosae and adherent mucus gel layers were eventually removed by gently scraping the surfaces with sterile scalpel blades and then placed on ice.

Composition and preparation of LHP medium

The new complex culture medium, LHP, was developed from five different components (solutions A-E) that were based on the descriptions of Rogosa *et al.* (1951) (SL medium; A), Hartemink *et al.* (1997) (LAMVAB medium, B and C), Marzotto *et al.* (2006) (C) and Juárez-Tomás *et al.* (2004) (D and E). The solutions were prepared individually and contained the following:

Solution A (968 ml^{-1}): trypticase or peptone from casein, 20.0 g; yeast extract, 10.0 g; glucose, 40.0 g; KH_2PO_4, 12.0 g; sodium acetate, 30.0 g; L-cysteine-HCl×H_2O, 1.12 g; Rogosa's salt solution, 10 ml; acetic acid, 2.64 ml. The pH was adjusted to 5.0 using 37-38 % HCl prior to autoclaving. Rogosa's salt solution (Rogosa *et al.*, 1951) contained the following (g 100 ml^{-1}): $MgSO_4$×$7H_2O$, 11.5; $MnSO_4$×$4H_2O$, 2.8; $FeSO_4$×$7H_2O$, 0.68.

Solution B (l^{-1}): agar, 40.0 g.

Solution C (ml^{-1}): vancomycin-HCl (Roth 0242), 25.0 mg.

Solution D (ml^{-1}): peroxidase from horseradish (Sigma P6782), 1.0 mg.

Solution E (ml^{-1} ≥99.8 % ethanol): TMB (Sigma T2885), 50 mg.

Solutions A and B were autoclave-sterilised at 100 °C for 10 min and 121 °C for 15 min, respectively. Solutions C and D were filter-sterilised using a 0.2-µl filter. Solutions A, C and D were sometimes prepared in advance and stored short-term at refrigeration temperature or long-term at -30 °C (C and D). Solutions B and E were always prepared fresh.

Preparation of 1 litre of LHP proceeded as follows: aliquots of solution A (484 ml), C (1.0 ml) and D (10 ml) were brought to room temperature. Solutions C and D were added to solution A. 500 ml of solution B were sterilised and cooled down to approximately 50 °C. 5 ml of warmed solution E (the ethanol had to be heated gently to dissolve the TMB) were added to solution B. Finally, solution A+C+D was added to solution B+E. Plates were poured immediately after thorough mixing. They were stored protected from light and were found to be stable for two weeks when kept at +4 °C.

The final concentrations of the ingredients in the complete LHP medium compared with Rogosa's SL, LAMVAB and MRS medium are listed in Table 1.

Table 1. Approximate composition (l^{-1}) of *Lactobacillus*-hydrogen peroxide (LHP) and other lactobacilli elective media

Medium	LHP	Rogosa's SL	LAMVAB	MRS
		(Merck 105413)	(Merck 110661)	(Roth X924)
	This study	Rogosa *et al.* (1951)	Hartemink *et al.* (1997)	de Man *et al.* (1960)
Peptone from casein	10.0 g	10.0 g	10.0 g	10.0 g
Yeast extract	5.0 g	5.0 g	4.0 g	4.0 g
Meat extract	-	-	8.0 g	8.0 g
Glucose	20.0 g	20.0 g	20.0 g	20.0 g
KH_2PO_4	6.0 g	6.0 g	-	-
K_2HPO_4	-	-	2.0 g	2.0 g
Tween 80	-	1.0 g	1.0 g	1.0 g
Ammonium citrate	-	2.0 g	2.0 g	2.0 g
Sodium acetate	15.0 g	15.0 g	5.0 g	5.0 g
Cysteine-HCl	0.5 g	-	0.25 g	-
$MgSO_4$	0.575 g	0.575 g	0.2 g	0.2 g
$MnSO_4$	0.14 g	0.14 g	0.04 g	0.05 g
$FeSO_4$	0.034 g	0.034 g	-	-
Acetic acid	1.32 ml	1.32 ml	-	-
Bromocresol green	-	-	0.025 g	-
Vancomycin-HCl	0.025 g	-	0.02 g	-
Peroxidase	0.01 mg	-	-	-
TMB	0.25 mg	-	-	-
Ethanol	5.0 ml	-	-	-
Agar	20.0 g	15.0 g	20.0 g	10.0 g

Isolation of lactobacilli and analysis of hydrogen peroxide accumulation

For the isolation and analysis of hydrogen peroxide on LHP medium, mucosal bacteria were washed off the sample surfaces using the following protocol: Aliquots of ≤1.0 g of the samples were transferred into 15 ml Greiner tubes (Greiner Bio-One) containing 1.0 g of sterilised glass beads (diameter 2.85–3.30 mm, Roth) and diluted 1:10 (samples from all three GI sections), 1:2 and 1:4 (jejunal samples) with ice-cold, sterile reduced phosphate-buffered saline (PBS) solution (l^{-1}: 8.0 g NaCl, 1.44 g Na_2HPO_4, 0.24 g NaH_2PO_4, 0.5 g L-cysteine; pH 7.4). The tubes were vigorously shaken using a Multi Reax shaker (Heidolph) for 10 min. 10-fold dilution series (10^{-2}-10^{-5}) were prepared from the 1:10 dilutions using the PBS solution in 1.5 ml Eppendorf tubes. Two 0.1-ml aliquots of each dilution were spread out on LHP agar plates by the use of Drigalski spatula.

The inoculated plates were treated following the description of Juárez-Tomás *et al.* (2004): They were incubated for 48 h at 37 °C under microaerobic [i.e. oxygen-reduced (5-7 % by vol.), CO_2-enriched (8-10 % by vol.)] conditions using Anaerocult C gas packs in anaerobic jars (both from Merck) and then exposed to air for 24 h at room temperature in the dark. Qualitative classification of the colony isolates as negative or positive for hydrogen peroxide accumulation was performed according to the description of Otero & Nader-Macías (2006). White colonies and colonies of any other (brown to blue) colour were evaluated as negative and positive, respectively. Viable cell counts were determined as the weighted average of colony-forming units (CFU) from two neighbouring dilutions using the formula of Farmiloe *et al.* (1954) (Bast, 2001). The rate of hydrogen peroxide accumulation was calculated as the mean percentage fraction of hydrogen peroxide-positive CFU from the total number of CFU.

Screening for manganese catalase by the catalase-benzidine dihydrochloride test

Colony isolates from LHP agar plates were screened for manganese catalase activity by using a qualitative catalase-benzidine dihydrochloride test on glass microscope slides. The procedure of the test was as follows:

An adequate mass of colony material is suspended with a disposable plastic inoculating loop in 1-2 µl of Tris-EDTA-sucrose (TES) lysis buffer containing 50 mM Tris-HCl, 1 mM EDTA and 25 % sucrose (Rochat *et al.,* 2006). 1-2 µl of 30 % H_2O_2 (VWR Prolabo 23612.294) is pipetted on the cell suspension and mixed using the pipette tip. The presence of catalatic activity leads to immediate vigorous effervescence due to the transformation of hydrogen peroxide to gaseous oxygen. If this occurs, then an equal volume (1-2 µl) of benzidine reagent solution containing (100 ml^{-1}) 1.0 g of benzidine dihydrochloride (Fluka 12125), 20 ml of glacial acetic acid and 50 ml of 95 % ethanol (Deibel & Evans, 1960) is added and mixed using the pipette tip. The presence of manganese catalase is indicated by no colour change, whereas a true catalase, due to the reaction of benzidine with haem, causes an immediate (within a few seconds) formation of a light turquoise to dark blue colour. The colouration is enhanceable by the addition of another 1-2 µl of 30 % H_2O_2. A slight decolouration after a few (ca. 2-5) minutes is normal.

Comparison of LHP and conventional complex media using mucosal bacterial isolates

In the course of a study of LARB from the mucosal stomach and proximal jejunum of one pig (Chapter IV) the obtained 27 bacterial isolates (16 of which were identified by means of partial 16S rRNA gene sequence analysis) were tested for growth on LHP, MRS (Roth X924), Slanetz and Bartley (SB) (Oxoid CM0377) and brain-heart-glucose (BHG) (Roth X915) agar plates. The strains were precultured anaerobically on MRS agar at 37 °C. From the

precultures, streaks were made on the aforesaid plate media, and the plates were incubated long-term under anaerobic conditions at 37 °C. Anaerobic [i.e. anoxic, CO_2-enriched (18 % by vol.)] conditions were achieved by using Anaerocult A gas packs in anaerobic jars (both from Merck). The '13-streak method' (Cypionka, 1999; Bast, 2001; Overmann, 2006) was always used for preparing streak cultures.

Validation of LHP medium and manganese catalase test using reference strains of LAB *sensu stricto*

The growth of reference strains of 25 recognised species of the genera *Lactobacillus* (*n*=20), *Enterococcus* (*n*=4; including *Ent. faecium* NCIMB 10415 from Cylactin) and *Streptococcus* (*n*=1) was evaluated on LHP medium. The strains were chosen based on the results of Leser *et al.* (2002). They represented, *inter alia*, the numerically predominant species of the LAB *sensu stricto* in the pig GI tract, namely *Streptococcus alactolyticus*, the overall most abundant phylotype according to Leser *et al.* (2002), and *L. amylovorus*, *L. johnsonii* and *L. reuteri*. *L. plantarum* CCUG 41414, which is known to a exhibit haem-independent catalase (http://www.ccug.se), was used as a positive control organism for the manganese catalase test (positive catalase and negative benzidine reaction). *Escherichia coli* DSM 2840 and *Bacillus subtilis* DSM 704 were used as controls for both a positive catalase and benzidine reaction.

The strains were precultured on MRS (lactobacilli), SB (enterococci) or BHG (other strains) agar plates for 48 h and then for 24 h under microaerobic conditions at 37 °C. Single colonies from the 24 h-precultures were streaked out in duplicate on LHP agar plates. An additional streak plate of the medium used for preculturing always served as control. The plates were incubated for 48 h under microaerobic conditions at 37 °C and then exposed to air for 24 h at room temperature in the dark. An evaluation of hydrogen peroxide accumulation was conducted generally as described above. According to the gradual colour intensity of positive colonies, a five-step evaluation system was used: a white or light brown, brown, light blue and dark blue colour was seen as indicative of negative or weak, moderate, strong and very strong, respectively, hydrogen peroxide accumulation. The manganese catalase test was carried out as already described. Colony material from the additional plate medium was used for the test if growth was negative on LHP. Strains of lactobacilli that were negative for growth on LHP medium under microaerobic conditions were retested using anaerobic conditions.

Results

Counts and hydrogen peroxide accumulation of isolates on LHP medium

Viable cell counts on LHP medium regularly were in the range of 2.9×10^3 to 7.1×10^7 CFU per 1 g of mucosal sample (Table 2). Within the GI tract of each individual pig, the viable counts tended to decrease from the oesophageal stomach to the proximal jejunum. In-feed addition of Cylactin containing the probiotic strain *Ent. faecium* NCIMB 10415 did not result in a notable increase of viable counts. The rate of hydrogen peroxide accumulation varied between approximately 50-98 %. In the 31-day-old pigs, the least rate occurred with isolates from the glandular stomach.

Table 2. Viable cell counts (VCC) and rate of hydrogen peroxide accumulation (HP) of isolates from pig gastric and jejunal mucosal samples on *Lactobacillus*-hydrogen peroxide medium

Pig			Stomach				Jejunum	
			oesophageal		glandular		proximal	
No.	Age (d)	Cylactin	VCC*	HP†	VCC	HP	VCC	HP
1	62	-	9.0×10^4	49.4	8.2×10^5	79.6	6.4×10^4	76.2
2	32	-	9.1×10^5	98.1	3.3×10^4	85.9	4.6×10^1	ND‡
3	31	-	1.0×10^5	98.1	2.0×10^4	82.7	2.9×10^3	96.4
4	31	-	7.1×10^7	67.4	1.3×10^6	60.3	1.1×10^5	93.9
5	31	+	2.3×10^7	98.3	1.2×10^6	66.1	2.8×10^4	94.3

*VCC, viable cell counts as weighted average of colony-forming units (CFU) from two neighbouring dilutions per 1 g of mucosal sample.

†HP, mean percentage fraction of hydrogen peroxide-positive CFU from the total number of CFU.

‡ND, not determined.

Manganese catalase of isolates on LHP medium

Colony material of a major number of hydrogen peroxide-negative and -positive isolates (n=≥68 and n=≥26; including pooled colonies) from different LHP agar plates was screened for the presence of manganese catalase activity using the combined catalase-benzidine dihydrochloride test. None of the material exhibited a positive catalase or benzidine reaction. Thus accordingly, no active manganese catalase was detected.

Growth of mucosal bacterial isolates on LHP and conventional complex media

MRS, SB and BHG medium supported the growth of all 27 isolates tested, including the strains lac15, lac16, lac31^T and lac18^T of the newly described species *Olsenella umbonata* and *Veillonella magna* (Table 3). The LHP medium proved to be limitedly sensitive and highly specific for lactobacilli. Seven of eight *Lactobacillus* strains and none of the strains of other genera were able to grow on LHP under anaerobic conditions at 37 °C. The *Lactobacillus* strain lac03 that was inhibited is most closely related to the species *L. equicursoris* Morita *et al.* 2010 of the phylogenetic *L. delbrueckii* group (Hammes & Hertel, 2009; Morita *et al.,* 2010).

Table 3. Growth of mucosal bacterial isolates on *Lactobacillus*-hydrogen peroxide (LHP) and conventional media under anaerobic conditions at 37 °C

Isolate	Phenotypically grouped	Most closely related species (% 16S rRNA gene sequence similarity)	LHP	MRS	SB	BHG
lac01		*Escherichia coli* (99.9)	No	Yes	Yes	Yes
lac30	lac10	*Escherichia coli* (99.8)	No	Yes	Yes	Yes
lac21	lac19/lac20/lac22	*Escherichia coli* (99.9)	No	Yes	Yes	Yes
lac13		*Bacillus anthracis* (99.5)	No	Yes	Yes	Yes
lac03		*Lactobacillus equicursoris* (98.5)	No	Yes	Yes	Yes
lac04		*Lactobacillus agilis* (98.5)	Yes	Yes	Yes	Yes
lac11	lac28	*Lactobacillus agilis* (98.0)	Yes	Yes	Yes	Yes
lac14	lac07/lac08/lac17	*Lactobacillus agilis* (98.5)	Yes	Yes	Yes	Yes
lac15		*Olsenella umbonata* sp. nov. (100)	No	Yes	Yes	Yes
lac16		*Olsenella umbonata* sp. nov. (100)	No	Yes	Yes	Yes
lac31^T		*Olsenella umbonata* sp. nov. (100)	No	Yes	Yes	Yes
lac27		*Enterococcus faecalis* (100)	No	Yes	Yes	Yes
lac09	lac05/lac06	*Enterococcus gallinarum* (99.8)	No	Yes	Yes	Yes
lac12	lac29	*Enterococcus gallinarum* (100)	No	Yes	Yes	Yes
lac32		*Enterococcus faecalis* (100)	No	Yes	Yes	Yes
lac18^T		*Veillonella magna* sp. nov. (100)	No	Yes	Yes	Yes

Growth was considered positive if colonies were visible. This included punctiform colonies.

Growth of LAB *sensu stricto* reference strains on LHP medium

Only lactobacilli exhibited growth on LHP medium (Table 4). Of the 22 strains of 20 *Lactobacillus* species included in the validation analysis, 16 strains of 15 species were able to grow on LHP agar plates under microaerobic conditions at 37 °C. Growth of members of the *L. delbrueckii* group, namely the species *L. amylovorus*, *L. johnsonii*, *L. delbrueckii* subsp. *delbrueckii*, *L. acidophilus* and *L. intestinalis*, was inhibited under both microaerobic and anaerobic conditions. The non-*Lactobacillus* LAB *sensu stricto* (i.e. *S. alactolyticus*, all enterococci) as well as *B. subtilis* and *E. coli* failed to grow on LHP medium.

Hydrogen peroxide accumulation of *Lactobacillus* reference strains on LHP medium and manganese catalase activity

Of the 16 *Lactobacillus* strains with positive growth on LHP medium, eight strains exhibited accumulation of hydrogen peroxide (Table 4). Accumulation was greatest among species from vaginal (*L. vaginalis*), oral (*L. salivarius*, *L. oris*) and gut mucosal (*L. mucosae*) origin. The results of the combined catalase-benzidine dihydrochloride test are also shown in Table 4. The catalase reaction was strongly positive for the control strains of *B. subtilis* and *E. coli* and also for the two strains of *L. plantarum*. For these latter strains, namely *L. plantarum* subsp. *plantarum* DSM 20174^{T} and *L. plantarum* CCUG 41414, this was a distinct capacity of the LHP medium, since the catalase reaction was always negative using colony material from the additional MRS agar plates. Both strains of *L. plantarum* did not accumulate hydrogen peroxide. The benzidine reaction was clearly positive (dark blue colouring) for *B. subtilis* and *E. coli*. For 13 of the 21 tested strains of LAB *sensu stricto*, a negative reaction (no colouring) was obtained. The type strains of *L. vaginalis*, *L. salivarius* and *L. fermentum* exhibited a weakly positive reaction (light blue colouring). With the strains of *L. plantarum* and three other *Lactobacillus* species (*L. reuteri*, *L. mucosae*, *L. agilis*), the benzidine reaction was uninterpretable due to a weak non-specific reaction (light blue colouring) that occurred upon addition of hydrogen peroxide. This reaction was not markedly enhanced by the subsequent addition of benzidine dihydrochloride. Taken the results of the catalase and benzidine reactions together, the type of catalase of *B. subtilis* and *E. coli* was, strictly speaking, not determinable, because these species are generally known to possess diverse haemoproteins (e.g. cytochromes). As to the strains of *L. plantarum*, the uninterpretable benzidine reactions impeded the identification of the detected catalases as manganese catalase.

Table 4. Growth and hydrogen peroxide accumulation of lactic acid-producing bacteria and other reference strains on *Lactobacillus*-hydrogen peroxide (LHP) medium and results of the catalase-benzidine dihydrochloride (manganese catalase) test

Strain	Growth*	H_2O_2†	Catalase reaction‡	Benzidine reaction‡	Manganese catalase‡
Streptococcus alactolyticus DSM 20728^T	No	ND	-	-	-
Lactobacillus amylovorus DSM 20531^T	No	ND	-	-	-
L. sobrius DSM 16698^T§	No	ND	-	-	-
L. johnsonii DSM 10533^T	No	ND	-	-	-
L. reuteri DSM 20016^T	Yes	++	-	NI	-
L. vaginalis DSM 5837^T	Yes	+++	-	(+)	-
L. sharpeae DSM 20505^T	Yes	++	ND	ND	ND
L. mucosae DSM 13345^T	Yes	+++	-	NI	-
L. plantarum subsp. *plantarum* DSM 20174^T	Yes	-	+	NI	NI
L. plantarum CCUG 41414	Yes	-	+	NI	NI
L. delbrueckii subsp. *delbrueckii* DSM 20074^T	No	ND	-	-	-
L. farciminis DSM 20184^T	Yes	-	ND	ND	ND
L. pontis DSM 8475^T	Yes	++	ND	ND	ND
L. panis DSM 6035^T	Yes	-	-	-	-
L. alimentarius DSM 20249^T	Yes	-	ND	ND	ND
L. salivarius subsp. *salivarius* DSM 20555^T	Yes	+++	-	(+)	-
L. ruminis DSM 20403^T	Yes	-	ND	ND	ND
L. fermentum DSM 20052^T	Yes	-	-	(+)	-
L. acidophilus DSM 20079^T	No	ND	-	-	-
L. rhamnosus DSM 20021^T	Yes	-	-	-	-
L. intestinalis DSM 6629^T	No	ND	-	-	-
L. oris DSM 4864^T	Yes	+++	ND	ND	ND
L. agilis DSM 20509^T	Yes	+	-	NI	-
Enterococcus faecium NCIMB 10415	No	ND	-	-	-
Enterococcus hirae DSM 20160^T	No	ND	-	-	-
Enterococcus faecalis DSM 20478^T	No	ND	-	-	-
Enterococcus gallinarum DSM 20628^T	No	ND	-	-	-
Bacillus subtilis DSM 704	No	ND	+	+	NI
Escherichia coli DSM 2840	No	ND	+	+	NI

*Growth was considered positive if colonies were visible. This included punctiform colonies.

†-, negative; +, moderate; ++, strong; +++, very strong. ND, not determined because no growth on LHP medium.

‡-, negative; (+), weakly positive; +, positive. NI, not interpretable. ND, not determined because not enough colony material. For strains with no growth on LHP medium, results of the catalase-benzidine dihydrochloride test are from cultures grown on MRS (lactobacilli), SB (enterococci) or BHG (other strains) medium.

§According to Jakava-Viljanen *et al.* (2008), *L. sobrius* is a later heterotypic synonym of *L. amylovorus*.

Discussion

LHP stands in the tradition of lactobacilli selective, complex acetate media (Sharpe, 1960). It is equivalent to LAMVAB medium, however based on Rogosa's SL medium and not on MRS as originally described (Hartemink *et al.*, 1997). Thus I intended to combine the improved lactobacilli elective (cysteine) and selective (vancomycin and low pH of 5.0 against non-*Lactobacillus* Gram-stain-positive and Gram-stain-negative bacteria, respectively) principals of LAMVAB with the superior qualities of Rogosa's SL medium [higher concentrations of magnesium and manganese for the stimulation of lactobacilli and increased sensitivity of hydrogen peroxide detection (Rabe & Hillier, 2003), iron and higher concentration of manganese for the stimulation of manganese catalase activity (Jones *et al.,* 1964; Johnston & Delwiche, 1965), acetic acid and higher concentration of acetate for the stimulation of growth and possibly hydrogen peroxide production of lactobacilli and for the suppression of other, mostly Gram-stain-negative bacteria (de Man *et al.,* 1960; Sharpe, 1960; Endo *et al.,* 2009; Gao *et al.,* 2009)]. LHP was adapted to its advanced application, the detection of hydrogen peroxide and manganese catalase, by addition of horseradish peroxidase and TMB and by omission of Tween 80 and ammonium citrate.

In the application of LHP on mucosal samples from the stomachs and jejuna of pigs, viable cell counts were between 10^3 and 10^7 CFU per 1 g of sample following microaerobic incubation for 48 h. This result is in the range of previously reported counts from pig ileal contents on anaerobically and longer (for three days) incubated Rogosa and LAMVAB agar (Hartemink & Rombouts, 1999), whereas the application of a *Lactobacillus*-specific 16S rDNA real-time polymerase chain reaction analysis on gastric and jejunal contents from 31-day-old pigs tended to result in higher counts (approx. 10^4-10^8 cells g^{-1}) (Klär, 2008). Considering that the growth of some lactobacilli is retarded at pH 5.2 (Sharpe, 1960), the pH measured in the uninoculated LHP medium post autoclaving, and stimulated by Tween 80 and citrate (de Man *et al.,* 1960; Gao *et al.,* 2009), both of which were not included in LHP, the obtained counts were relatively high. Particularly in view of the microaerobic conditions used, this might be due to the elevated concentration of manganese ions and the presence of horseradish peroxidase. High intracellular manganese can confer enhanced oxidative stress resistance in the LAB *sensu stricto* by superoxide dismutase-like detoxification of superoxide radicals and prevention of the hydroxyl radical-generating Fenton reaction (Archibald & Fridovich, 1981; Archibald, 1986; Barnese *et al.,* 2008; Imlay, 2008). The addition of horseradish peroxidase to MRS medium has been shown to sustain the growth of hydrogen peroxide-accumulating lactobacilli (Sakamoto *et al.,* 1998).

Supplementation of Cylactin containing the probiotic strain *Ent. faecium* NCIMB 10415 did not result in an increase of viable cell counts. This indicates that, due to its high specificity, the new LHP medium is suitable for application in feeding studies with non-*Lactobacillus* probiotic LAB. LHP is highly specific for lactobacilli in so far as, in the validation analysis, none of the 26 isolate and reference strains of the genera *Enterococcus* (*n*=11), *Streptococcus* (*n*=1), *Bacillus* (*n*=2), *Veillonella* (*n*=1), *Olsenella* (*n*=3) and *Escherichia* (*n*=8) were able to grow on LHP under anaerobic or microaerobic conditions. This group specificity of LHP contrasts the general applicability of the other media tested (MRS, SB and BHG).

Non-inhibition of probably vancomycin-resistant faecal enterococci and other non-*Lactobacillus* LAB *sensu stricto* (streptococci, pediococci) has been realised as a major limitation of LAMVAB medium (Hartemink *et al.*, 1997). Vancomycin resistance of enterococci is coded by a transferable plasmid (Mishra *et al.,* 2009). It has been reported in Europe with increasing prevalence since 1986 (Devriese *et al.,* 2006; Leblanc, 2006) and is more widespread among strains from domestic pigs (Kühn *et al.,* 2005; Manero *et al.,* 2006) than from wild pigs (Poeta *et al.,* 2007). Horizontal conjugative transfer of vancomycin resistance within microbial food chains containing enterococci, other LAB and potentially veillonellae is of significant concern, as vancomycin is the last resort antibiotic in the treatment of infections with multiresistant staphylococci (Axelsson, 2004; Carlier, 2009; Mishra *et al.,* 2009). LAMVAB contains 20 mg vancomycin-HCl l^{-1}. With the new LHP medium, a concentration of 25 mg vancomycin-HCl l^{-1} was used according to the description of MRS-Van medium for faecal lactobacilli (Marzotto *et al.*, 2006). This higher concentration of vancomycin in LHP might be responsible for the observed total inhibition of enterococci and other non-*Lactobacillus* Gram-stain-positive bacteria.

In total, 30 strains of 21 species of lactobacilli were analysed for growth on LHP. Seven strains of six species of the phylogenetic *L. delbrueckii* group, namely *L. amylovorus*, *L. johnsonii*, *L. delbrueckii* subsp. *delbrueckii*, *L. acidophilus*, *L. intestinalis* and, presumably, *L. equicursoris* (isolate lac03) failed to grow under both microaerobic and anaerobic conditions. This inhibition of all tested strains of the *L. delbrueckii* group is most likely due to their susceptibility to vancomycin. Many lactobacilli exhibit intrinsic resistance to vancomycin due to the presence of a D-Ala-D-lactate depsipeptide in place of D-Ala-D-Ala dipeptide in the cell wall peptidoglycan layer (Lebeer *et al.,* 2008; Hammes & Hertel, 2009; Mishra *et al.,* 2009; Kleerebezem *et al.,* 2010). Vancomycin susceptibility of lactobacilli is species-related, as it is characteristically more frequent among members of the *L. delbrueckii* group (Hamilton-Miller & Shah, 1998; Felten *et al.,* 1999). However, it is strain-specific in so far as, using LAMVAB agar, inhibition has been reported by Jackson *et al.* (2002) (*L. acidophilus*

isolates from human faeces) and Dal Bello *et al.* (2003) (*L. delbrueckii* subsp. *bulgaricus* DSM 20081^T), however not by Hartemink *et al.* (1997) (*L. acidophilus* isolates from dairy starter and pig faeces, strain of *L. delbrueckii* subsp. *bulgaricus*).

A limited sensitivity for lactobacilli due to vancomycin susceptibility of strains of the *L. delbrueckii* group remains a drawback of the new LHP medium. The impact of this drawback depends on the respective habitat and source of isolation. In the GI tract of pigs, both *L. amylovorus* and *L. johnsonii* seem to be abundant, whereas *L. delbrueckii*, *L. acidophilus* and *L. intestinalis* are quantitatively less important (Leser *et al.*, 2002; Klär, 2008). According to Hammes & Hertel (2009), these five *Lactobacillus* species are all associated with the GI tracts of humans, other mammals and birds.

The rate of detection of hydrogen peroxide in qualitative culture-based assays significantly depends on the composition of the culture medium (Rabe & Hillier, 2003; Zalán *et al.,* 2005). In the practical application of LHP, the rate of hydrogen peroxide accumulation varied between approximately 50 and 98 %. This rather high rate is generally consistent with the results of other studies on vaginal or intestinal lactobacilli using TMB (Felten *et al.,* 1999; Mota *et al.,* 2006; Otero & Nader-Macías, 2006). It is likely to be attributable to the special composition of LHP, i.e. the absence of known inhibitory substances (haem, Tween 80, citrate) and increased amounts of enhancive ingredients (magnesium, manganese, acetic acid/acetate). The determined rate of hydrogen peroxide accumulation might deviate from the rate of production due to catalatic activity of many isolates. However, as none of the colony isolates tested exhibited a positive catalase reaction in the catalase-benzidine dihydrochloride test, this was apparently not the case.

No active manganese catalase was detected during the screening of a major number of hydrogen peroxide-negative colony isolates from different LHP agar plates. This confirms Engesser & Hammes (1994) who observed the non-haem catalase in strains of only three of 71 species of the LAB *sensu stricto* (*L. plantarum*, *L. mali* and *Pediococcus pentosaceus*) and thus stated that its occurrence is rare compared with the haem-dependent catalase in the presence of exogenous haematin (21 species found positive). According to the results of Jones *et al.* (1964), Johnston & Delwiche (1965a) and Engesser & Hammes (1994), the manganese catalase is sufficiently active at low pH of 4.5-5.5 and at both room temperature and 37 °C, so it is less probable that the activity was inhibited or reduced to non-detectable levels in my study. On the contrary, unlike MRS the new LHP medium facilitated the detection of catalase activity in both *L. plantarum* subsp. *plantarum* DSM 20174^T and *L. plantarum* CCUG 41414.

The non-haem catalase has been commonly described in the recognised reference strain *L. plantarum* T-1403-5 =ATCC 14431 and also in a few other isolate strains of *L. plantarum* (Dacre & Sharpe, 1956; Langston & Bouma, 1960; Johnston & Delwiche, 1962; Johnston & Delwiche, 1965a), but not in the type strain *L. plantarum* ATCC 14917^T =DSM 20174^T (Yousten *et al.,* 1975; Archibald, 1986; Bringel *et al.,* 2005; Hammes & Hertel, 2009). To my knowledge, the study of this chapter is the first to report an active haem-independent catalase in the type strain of *L. plantarum* subsp. *plantarum*. The fact that both *L. plantarum* subsp. *plantarum* DSM 20174^T and *L. plantarum* CCUG 41414 did not accumulate hydrogen peroxide confirms the hypothesis that the screening of hydrogen peroxide-negative colony isolates using the catalase-benzidine dihydrochloride test presents an effective approach for the detection of manganese catalase of strains of potentially novel species of *Lactobacillus*.

Jones *et al.* (1964) and Johnston & Delwiche (1965) noted that manganese and iron in growth media stimulated the haem-independent catalase activity. A high intracellular level of manganese is essential for the activity of manganese catalase, since this enzyme is a hexamer with two manganese atoms per subunit (Beyer & Fridovich, 1985; Barynin *et al.,* 2001). Internal manganese accumulation in lactobacilli and other LAB *sensu stricto* is likely to be of ecological relevance in the rumen and other GI habitats in which manganese is sufficiently available from plant cell material or following dietary supplementation (Archibald, 1986; Axelsson, 2004). Addition of iron to complex media for lactobacilli is not necessary generally, because as other LAB *sensu stricto* lactobacilli have no explicit requirement for iron (MacLeod & Snell, 1947; de Man *et al.,* 1960; Vandevoorde *et al.,* 1992). However, intracellular iron imposes oxidative stress through stimulation of NADH oxidase and the hydroxyl radical-generating Fenton reaction (Archibald, 1986; Bruno-Bárcena *et al.,* 2004; Imlay, 2008) and thus might positively interfere with manganese catalase activity.

In many studies, it has been found that the manganese catalase is more active using glucose minimal media (Felton *et al.,* 1953; Dacre & Sharpe, 1956; Jones *et al.,* 1964; Engesser & Hammes, 1994), and occasionally using 1 % glucose no activity was detected in strains of *L. plantarum* (Deibel & Evans, 1960). This effect might be in part due to the glucose catabolite repression of the production of hydrogen peroxide, a catalase-inducing agent, via flavoprotein oxidases (Condon, 1987; Hertel *et al.,* 1998). In the present study, the new LHP medium as the other lactobacilli elective media (Rogosa's SL, LAMVAB, MRS) contained 2 % glucose. According to Rabe & Hillier (2003), qualitative detection of hydrogen peroxide should be attempted only if adequate growth is apparent, and therefore the concentration of glucose in culture media must not be too low. The catalase reactions of the strains of *L. plantarum* were strongly positive despite 2 % glucose, presumably because the main role of NADH:H_2O_2

oxidase, which presents the principal constitutive hydrogen peroxide-producing oxidase of the LAB *sensu stricto* under microaerobic conditions, is detoxification, i.e. elimination of molecular oxygen, and not NAD^+ regeneration (Murphy & Condon, 1984; van de Guchte *et al.,* 2002).

The benzidine dihydrochloride test was originally introduced for the routine detection of cytochrome-containing respiratory systems in bacteria (Deibel & Evans, 1960). At that time, before the non-haem, manganese nature of the 'pseudocatalase' (Whittenbury, 1964) was identified (Delwiche, 1961; Kono & Fridovich, 1983), this test was supposed to be superior to the catalase test for differentiating the LAB *sensu stricto* from morphologically similar cytochrome-containing bacteria, because 'it is not confused by the presence of trace levels of catalase' of the LAB (Deibel & Evans, 1960). Hence Deibel & Evans (1960) obtained a negative benzidine reaction with two catalase-positive strains of *L. plantarum*. They actually detected the non-haem manganese catalase but, at their time, were not able to recognise the implication of this result.

In the catalase-benzidine dihydrochloride test of this study, the application of TES lysis buffer is useful, because manganese catalase is a cytoplasmic enzyme that is not secreted into the culture medium (Rochat *et al.,* 2006). However, the catalase reactions of both *L. plantarum* subsp. *plantarum* DSM 20174^T and *L. plantarum* CCUG 41414 were positive also without prior addition of TES, so it is not mandatory. Deibel & Evans (1960) used a 5 % H_2O_2 solution in their benzidine dihydrochloride test. Following the description of Rochat *et al.* (2006), I used a 30 % H_2O_2 solution. A higher concentration is suitable to compensate for the dilution effect of TES buffer, for the low affinity of catalase for hydrogen peroxide (Percy, 1984) and also for catalase degradation of hydrogen peroxide prior to the reaction with benzidine.

The weak non-specific reactions of, *inter alia*, the reference strains of *L. plantarum* were probably due to the interference of hydrogen peroxide with peroxidase and TMB in the culture medium. The observed weakly positive benzidine reactions of other *Lactobacillus* strains might have been delayed non-specific reactions or indicative of small amounts of endogenous haem. Whittenbury (1964) detected traces of presumably endogenous haem or cytochrome in manganese catalase-negative strains of *L. plantarum*. Also, an endogenously active nitrate reductase has been noticed in *L. plantarum* by Wolf & Hammes (1988).

The identification of the type of catalase in the two strains of *L. plantarum* was impeded by the non-specific reactions. A simultaneous occurrence of the manganese catalase and true catalase has been reported after growth of lactobacilli with a source of haem (Johnston & Delwiche, 1965), however since LHP does not contain any haem compounds, the expression of an active haem-dependent catalase by lactobacilli from this medium is highly improbable. Therefore and due to the high specificity for lactobacilli, a catalase of an unidentified *Lactobacillus* isolate from LHP is in all probability a manganese catalase and the isolate a strain of either *L. plantarum*, *L. mali* or a novel *Lactobacillus* species.

Overall, the new LHP medium was validated as highly specific for lactobacilli and thus is applicable for direct isolation of these bacteria from the complex microbiota of the pig GI tract, even in feeding studies using non-*Lactobacillus* LAB as probiotics. However, as LAMVAB, LHP is limited in its sensitivity and ability to give an accurate representation of lactobacilli due to the vancomycin susceptibility of members of the *L. delbrueckii* group. The use of vancomycin as selective agent in primary isolation media for gut lactobacilli is generally questioned. LHP can be recommended for the qualitative analysis of hydrogen peroxide accumulation of microaerobically grown lactobacilli. It is suitable for the detection of the haem-independent manganese catalase, such as in strains of *L. plantarum* subsp. *plantarum*. In combination with a screening catalase-benzidine dihydrochloride test on hydrogen peroxide-negative colony isolates, LHP constitutes a simple approach for the detection of strains of potentially novel *Lactobacillus* species.

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Chapter IV

PGM-plus and PGM: new mucin-based media in the specific isolation of lactic acid-related bacteria from the mucosal stomach and jejunum of a pig, including *Olsenella umbonata* sp. nov. and *Veillonella magna* sp. nov.

Introduction

In the proximal gastrointestinal (GI) tract of pigs and other monogastric mammals, complex mucin glycoproteins present the principal macromolecular structural components of the inner epithelial adherent mucus gel layer, which is a prevailing microhabitat for the timely permanent or resident indigenous microbiota (Allen & Snary, 1972; Collinder *et al.,* 2003; Pearson & Brownlee, 2005; Linden *et al.,* 2008).

The mucin carbohydrate side chains are degradable only by specific bacterial glycosidases, since only unspecific and own their own ineffective proteases of the animal hosts exist (Hoskins, 1992; Collinder *et al.,* 2003). Therefore, in monogastric mammals, endogenous mucins likely exert an ecological role in the proximal GI tract similar to plant-derived complex carbohydrates in the lumen of the distal large intestine.

Effective exploitation of the large, polymeric and heterogeneous gel-forming mucin populations (Gum, 1995; Nordman *et al.,* 1998; Linden *et al.,* 2008) requires the concerted and complementary actions of a variety of specific glycosidic and non-specific proteolytic enzymes from diverse cooperative bacterial species (Hoskins, 1992; Bradshaw *et al.*, 1994; Wickström *et al.,* 2009). Thus, due to their inherent multiple and diverse metabolic sites (niches), mucins in the inner mucus gel layer accomplish an ecologically outstanding stabiliser function. They present a continuously supplied matrix for maintenance of a highly diverse and hence more resilient and stable mucosa-associated bacterial community, particularly in the interdigestive proximal GI tract (Hoskins *et al.,* 1985; Lee, 1985; Hoskins, 1992; Deplancke *et al.,* 2002; Laux *et al.,* 2005; Little *et al.,* 2008).

In the hand of the bacteriologist, who attempts to recover from the GI mucosae a bacterial community that is naturally complex and in its diversity distinct from the luminal counterpart

(Savage, 1977), commercially available mucin, such as porcine gastric mucin (PGM) (=mucin from porcine stomach; Sigma M1778), is a promising basal component for the development of a new isolation medium. A nutritionally minimal version of a PGM-based culture medium has successfully been applied by Derrien *et al.* (2004) in the elective isolation of a novel and apparently markedly mucin oligosaccharide degrading species (Hoskins, 1992), namely *Akkermansia muciniphila*, from human faeces. PGM is orthologous to human mucins from the oral cavity and colon (Bradshaw *et al.*, 1994; Macfarlane *et al.*, 2005).

PGM is suitable for the isolation of lactic acid-related bacteria (LARB), since lactic acid is an intermediate product from the bacterial degradation and fermentation of the heavily glycosylated mucin proteins (Bradshaw *et al.*, 1994; Laux *et al.,* 2005). Elective and selective agents are mandatory for the specific isolation of these bacteria from the mucosae of the pig proximal GI tract, where other microorganisms, such as Gram-stain-negative enterobacteria, are numerous (Leser *et al.,* 2002). Growth of the facultatively or aerotolerant anaerobic LAB *sensu stricto*, mainly of lactobacilli, is traditionally favoured by using microaerobic culture conditions and high supplementation of manganese and magnesium (Sharpe, 1960; Hammes & Hertel, 2006). Their selection against Gram-stain-negative enterobacteria is accomplished by using a low pH as the key factor (Sharpe, 1960). The LAB *sensu lato* of the *Actinobacteria* and the metabolically linked, lactic acid-fermenting veillonellae and megasphaerae are typically oxygen-sensitive obligate anaerobes and fastidious, exhibiting such as bifidobacteria slow growth on PGM (Macfarlane *et al.,* 2005) or relying on lactic acid from the prior fermentation of monosaccharides of the degraded mucin glycan side chains. These groups of LARB require anaerobic culture conditions and a sufficiently long time for propagation.

The objectives of the present study on the microbiota of the pig mucosal stomach and jejunum were to: *i*) specifically isolate a complex, phylogenetically diverse and partially novel community of LARB using PGM as basal component of a newly developed culture medium; and *ii*) identify the isolated bacterial community by means of a 16S rRNA gene sequence-based polyphasic taxonomic approach.

Materials and methods

Pig

A cross-bred (Euroc×Piétrain) domestic pig (*Sus scrofa domestica*) was used. The pig was born and reared at the Institute of Animal Nutrition during the approved scientific study

'Integrative analysis of the modes of action of probiotics in pigs' (German Research Foundation research group FOR 438). It was weaned at four weeks of age and from a control group that had not received any probiotic feed. It ingested approx. 1.34-3.35 g chromium oxide (Cr_2O_3) per day in the course of a digestibility study for six days ante mortem (data from D. Taras, unpublished results). The pig was euthanized at 62 days of age, weighed 25.9 kg and was clinically healthy at this day.

Gutting and sampling

These steps were performed essentially as described previously (Chapter III).

Composition and preparation of PGM and PGM-plus

The new mucin-based culture medium was used in two variants, PGM-plus and PGM. These contained (per litre) the following components of an unsupportive basal medium (UBM): NaCl, 0.3 g; $CaCl_2$, 0.1 g; KH_2PO_4, 6.0 g; Rogosa's salt solution, 5 ml; trace element solution, 1 ml; vitamin solution, 0.2 ml; resazurin, 0.5 mg. Rogosa's salt solution (Rogosa *et al.*, 1951) contained the following (g 100 ml^{-1}): $MgSO_4 \times 7H_2O$, 11.5; $MnSO_4 \times 4H_2O$, 2.8; $FeSO_4 \times 7H_2O$, 0.68. The trace element solution was a modified version (lacking manganese and iron) of trace element solution SL 8 (Pfennig & Trüper, 1981; Bast, 2001). The vitamin solution was based on the description of Stams *et al.* (1993) and contained the following (g l^{-1}): biotin, 0.02; nicotinic acid, 0.2; pyridoxal-HCl, 0.5; riboflavin, 0.1; thiamine-HCl, 0.2; cyanocobalamin, 0.1; p-aminobenzoic acid, 0.1; Ca-pantothenate, 0.1; folic acid; 0.002. One litre of the media was prepared by mixing all of the UBM components, except the vitamin solution, with 0.01 g each of peptone, yeast extract and glucose (PYG) (PGM-plus), 10.0 g of mucin from porcine stomach (Sigma M1778) (PGM-plus and PGM) and distilled water to 1000 ml. For the initial enrichment of bacteria, the PGM-plus was used as a liquid medium. For later isolation steps PGM-plus and then PGM media containing 0.4-0.75 % agar were prepared. The pH of the media was adjusted to 5.0±0.1 using 37-38 % HCl. The media were autoclaved at 100 °C for 10 min and cooled down to approximately 50 °C. The vitamin solution was sterilised using a 0.2-µm filter and added to the cooled media. Plates were poured immediately after thorough mixing. They were stored at +4 °C and protected from light.

Isolation of mucosal bacteria

The isolation of mucosal bacteria proceeded in four major steps: *i*) enrichment in liquid PGM-plus; *ii*) cultivation in serial dilutions in liquid PGM-plus; *iii*) sub-culturing on semi-solid (0.75 % agar) PGM-plus and soft (0.4 % agar) PGM; and *iv*) isolation of pure cultures on conventional complex media.

From each of the three GI sections (oesophageal and glandular stomach, proximal jejunum), three pinpoint mucosal samples were transferred into separate 1.5 ml Eppendorf tubes containing 1.0 ml of liquid PGM-plus. The tubes were vigorously mixed using a Vortex-Genie 2 (Scientific Industries), and two 0.5-ml aliquots from each tube were inoculated to 10 ml of liquid PGM-plus in loosely screw-capped 15 ml Greiner tubes (Greiner Bio-One). One aliquot assay of each sample was incubated for 14 days at 37 °C under anaerobic [i.e. anoxic, CO_2-enriched (18 % by vol.)] conditions and the other one under microaerobic [i.e. oxygen-reduced (5-7 % by vol.), CO_2-enriched (8-10 % by vol.)] conditions using Anaerocult A and Anaerocult C gas packs, respectively, in anaerobic jars (all from Merck). Always the same atmospheric condition (anaerobic or microaerobic) was used for the subsequent steps of isolation. 100 µl of each of the three parallel tubes (of the same GI section and atmospheric condition) were then pooled in a fresh 10-ml volume of liquid PGM-plus and incubated for another seven days. Thereafter, 10-fold dilution series were prepared in 9.0 ml of sterile 0.9 % reduced (containing 0.5 g L-cysteine-HCl l^{-1}) NaCl solution, 1 ml of the 10^{-3} to 10^{-8} dilutions were inoculated into 9.0 ml of liquid PGM-plus (10^{-4} to 10^{-9}) and incubated for seven to ten days. The tubes were macroscopically and microscopically checked for evidence of growth (cell sediment, discolouration of resazurin and cell density). Starting from tubes of the highest dilution with good growth, two 10-fold dilutions were prepared in sterile 0.9 % reduced NaCl solution, and heavy inocula (500 µl aliquots) from the undiluted tube contents and the dilutions were spread out on semi-solid PGM-plus plates by the use of Drigalski spatula. The plates were incubated for seven days. Detailed cell morphological studies of the obtained cultures were performed using a Zeiss light microscope at × 1000 magnification. Colonies of all distinguishable morphotypes were streaked on plates of soft PGM and incubated for 14 days. Thus eventually bacterial isolates of distinct cell morphotypes were obtained in pure culture on de Man-Rogosa-Sharpe (MRS) (Roth X924), Slanetz and Bartley (SB) (Oxoid CM0377) and brain-heart-glucose (BHG) (Roth X915) agar streak plates. The ‘13-streak method’ (Cypionka, 1999; Bast, 2001; Overmann, 2006) was always used as the way of preparing streak cultures.

Initial phenotypic analysis

With the aim of further assigning the cell morphotypes to major phenotypic groups, the isolates were subjected to basic morphological, cultural, physiological and biochemical analyses. Morphological studies included colony morphology on MRS agar, Gram-staining with a Gram-colour staining set (Merck) and spore tests (Bast, 2001). Cultural and physiological analyses were carried out by observation of anaerobic growth on different complex media [*Lactobacillus*-hydrogen peroxide (LHP), MRS, SB, BHG (Chapter III)] as well as of growth at different temperatures (21 and 45 °C) and oxygen conditions (aerobic and microaerobic) on MRS agar. Biochemical analyses comprised a cytochrome oxidase test for Gram-stain-negative bacteria (Fluka 70439), the catalase-benzidine dihydrochloride test (Chapter III) and the VITEK system with Anaerobe Identification (ANI) cards (bioMérieux). For the latter analysis, BHG agar medium was chosen for propagation of the isolates.

16S rRNA gene sequence-based phylogenetic analysis

For subsequent phylogenetic analysis, genomic DNA was extracted from cells of representative strains of each phenotypic group using a NucleoSpin Tissue kit (Macherey-Nagel). The almost complete 16S rRNA gene was amplified by polymerase chain reaction (PCR) in a T1 Thermocycler (Biometra) using a HotStarTaq Master Mix kit (Qiagen) and the universal bacterial primer pair 27f and 1492r (Lane, 1991) or, with regard to the isolates lac15, lac16, lac31^T and A2, also the *Coriobacteriaceae* suited primers C75 and C90 (Dewhirst *et al.,* 2001). The PCR amplicons were purified using a High Pure PCR Product Purification kit (Roche). The product purity was checked by electrophoresis in a 1.5 % agarose gel, and the final DNA yield was quantified using a NanoDrop ND-3300 fluorospectrometer (Peqlab) in combination with the dye bisbenzimide (Hoechst 33258, Bio-Rad). Partial (849-1449 bp) nucleotide sequences were determined directly from the purified PCR products with primers 27f and 1492r or C75 and C90 by the sequencing services of Eurofins MWG Operon. An assignment of the gene sequences to taxonomic genera was performed with the online (http://rdp.cme.msu.edu/classifier) Ribosomal Database Project (RDP) II classification algorithm (Cole *et al.,* 2009). The similarities to sequences of closely related recognised strains were determined using the BLASTn algorithm (Johnson *et al.,* 2008) of the NCBI web server (http://blast.ncbi.nlm.nih.gov). An alignment of the newly determined sequences and eight closely related sequences retrieved from GenBank was carried out with the CLUSTAL W tool (Thompson *et al.,* 1994) implemented in MEGA software version 4.1 (Tamura *et al.,* 2007). Also, in MEGA4, a phylogenetic tree was constructed by using the unweighted pair-group method with arithmetic mean (UPGMA) (Sokal & Michener, 1985). The UPGMA tree was inferred from pairwise evolutionary distances of the aligned sequences that had been computed using the maximum composite

likelihood method (Tamura *et al.,* 2004) with the Tamura-Nei nucleotide substitution model (Tamura & Nei, 1993) after complete deletion of positions with gaps or missing data. Statistical reliability of the UPGMA tree was tested by the bootstrap method (Felsenstein, 1985) with 1000 replicates.

Results

Phenotypic groups of mucosal bacteria

A total of 27 bacterial isolates of five different cell morphotypes (plump rod, long thin rod, small irregular rod, coccoid rod, small coccus) were obtained in pure culture from the gastric and jejunal mucosal samples. All of the isolates were able to grow on MRS, SB and BHG agar plates under anaerobic conditions at 37 °C, whereas the new LHP medium specifically supported the growth of all long thin rods except strain lac03 (Chapter III). Colonies on PGM-plus and PGM agar plates were weak and highly uniform among the isolates, however using MRS agar plates the strains could be macroscopically differentiated. Based on cellular morphology and oxygen relationship, the isolates were further assigned to seven major phenotypic groups (Table 1).

Table 1. Results of the phenotypic analysis of mucosal bacterial isolates assigned to seven major phenotypic groups

Strain	Isolation source/method*	Growth on MRS at		Oxidase†	Catalase†	Mucinolytic glycosidases‡ (VITEK ANI)				
		21/45 °C	5-7 % O_2			AFUC	AGAL	BGAL	NAG	AMAN
Plump rod, Gram-stain-negative, non-spore-forming, facultatively anaerobic										
lac01	SO/anaerobic	No/Yes	Yes	-	+	-	+	+	a	-
lac30	SG/microaerobic	No/Yes	Yes	-	+	-	+	+	a	-
lac10	SG/microaerobic	No/Yes	Yes	ND	+	-	+	+	+	-
lac21	J/microaerobic	No/No	Yes	-	+	-	+	+	+	-
lac19	J/microaerobic	No/Yes	Yes	ND	+	-	+	+	a	-
lac20	J/microaerobic	No/No	Yes	ND	+	ND	ND	ND	ND	ND
lac22	J/microaerobic	No/Yes	Yes	ND	+	ND	ND	ND	ND	ND
Plump rod, Gram-stain-positive, spore-forming, facultatively anaerobic										
lac13	SG/microaerobic	No/No	Yes	ND	+	-	-	-	-	-
Long thin rod, Gram-stain-positive, non-spore-forming, aerotolerant anaerobic										
lac03	SO/microaerobic	No/Yes	Yes	ND	-	-	+	-	-	-
Long thin rod, Gram-stain-positive, non-spore-forming, microaerotolerant (5-7 % O_2) (moderately obligate) anaerobic										
lac04	SO/microaerobic	No/Yes	Yes	ND	-	-	+	+	+	-
lac07	SG/anaerobic	No/Yes	Yes	ND	-	-	+	+	-	-
lac08	SG/anaerobic	No/Yes	Yes	ND	-	ND	ND	ND	ND	ND
lac11	SG/microaerobic	No/Yes	Yes	ND	-	-	+	+	-	-
lac28	SG/microaerobic	No/Yes	Yes	ND	-	ND	ND	ND	ND	ND
lac14	J/anaerobic	No/Yes	Yes	ND	-	-	+	+	-	-
lac17	J/anaerobic	No/Yes	Yes	ND	-	ND	ND	ND	ND	ND

Strain	Isolation source/method*	Growth on MRS at		Oxidase†	Catalase†	Mucinolytic glycosidases‡ (VITEK ANI)				
		21/45 °C	5-7 % O_2			AFUC	AGAL	BGAL	NAG	AMAN
Small irregular rod, Gram-stain-positive, non-spore-forming, microaerotolerant (< approx. 5% O_2) (moderately obligate) anaerobic										
lac15	J/anaerobic	No/Yes	No	-	-	-	-	-	-	-
lac16	J/anaerobic	No/Yes	No	-	-	-	-	-	-	-
lac31^T	J/anaerobic	No/Yes	No	-	-	-	-	-	-	-
Coccoid rod, Gram-stain-positive, non-spore-forming, aerotolerant anaerobic										
lac27	SO/microaerobic	Yes/Yes	Yes	ND	-	-	+	+	+	-
lac09	SG/anaerobic	Yes/Yes	Yes	ND	-	-	+	+	+	-
lac05	SG/anaerobic	Yes/Yes	Yes	ND	-	ND	ND	ND	ND	ND
lac06	SG/anaerobic	Yes/Yes	Yes	ND	-	ND	ND	ND	ND	ND
lac12	SG/microaerobic	Yes/Yes	Yes	ND	-	-	a	+	+	-
lac29	SG/microaerobic	Yes/Yes	Yes	ND	-	ND	ND	ND	ND	ND
lac32	J/anaerobic	Yes/Yes	Yes	ND	-	-	+	+	+	-
Small coccus, Gram-stain-negative, non-spore-forming, microaerotolerant (< approx. 5% O_2) (moderately obligate) anaerobic										
lac18^T	J/anaerobic	No/Yes	No	-	- or (+)	-	-	-	-	-

*SO, oesophageal stomach; SG, glandular stomach; J, proximal jejunum.

†-, negative; +, positive; (+), weak; ND, not determined.

‡AFUC, *α*-fucosidase; AGAL, *α*-galactosidase; BGAL, *β*-galactosidase; NAG, *N*-acetyl-glucosaminidase; AMAN, *α*-mannosidase; a, ambiguous.

Names of strains that were used as representatives for 16S rRNA gene sequence-based phylogenetic analysis are in bold.

Strains of all groups were mesophilic, i.e. under anaerobic conditions they produced no or, compared with 37 °C, impaired growth at 21 and 45 °C. Only the coccoid rods grew at 21 °C. Furthermore, all of the strains were able to either use or tolerate molecular oxygen. 16 strains exhibited positive (lac03, coccoid rods) or even enhanced (plump rods) growth under aerobic conditions. The seven isolated long thin rods (except strain lac03) were intolerant to atmospheric oxygen but exhibited good growth under microaerobic, oxygen-reduced conditions. The strains lac15, lac16 and lac31^T, three small irregular rods, and also strain lac18^T, a small coccus, failed to grow on agar plates under conditions of 5-7 % oxygen. However, during further phenotypic studies they were found to be capable of slightly impaired but good (lac15, lac16 and lac31^T) or unimpaired (lac18^T) growth in microaerobic URAS (unreduced aerobically sterilised) liquid media (Chapters V and VI). The cytochrome oxidase test did not serve to differentiate among the isolates because, to the extent performed, it was always negative. In the catalase-benzidine dihydrochloride test, only the facultatively anaerobic rods exhibited a positive catalase reaction. The reaction of strain lac18^T was non-specific, as it was at most delayed and weak. Mucinolytic glycosidases were ubiquitously present in the majority of the isolates analysed. Only five strains (lac13, the small irregular rods and lac18^T) showed no positive reaction. Seven strains produced two and six strains, mostly the coccoid rods, produced three glycosidases. α- and β-galactosidase were more frequently (n=13 each) positive than *N*-acetyl-glucosaminidase (n=7). No positive α-fucosidase or α-mannosidase was detected.

Phylogenetic relationships of identified isolates

The results of the 16S rRNA gene sequence-based phylogenetic analysis of 16 representative strains revealed that the isolated bacterial community comprised six different genera (Table 2).

Table 2. Results of 16S rRNA gene sequence-based identification of representative isolates of each phenotypic group

Strain	Phenotypically grouped	RDP genus classification	Most closely related recognised strain	% sequence similarity
lac01		*Escherichia*	*Escherichia coli* ATCC 8739	99.9
lac30	lac10	*Escherichia*	*Escherichia coli* ATCC 8739	99.8
lac21	lac19/lac20/lac22	*Escherichia*	*Escherichia coli* ATCC 8739	99.9
lac13		*Bacillus*	*Bacillus anthracis* CDC 684	99.5
lac03		*Lactobacillus*	*Lactobacillus equicursoris* DI70^T	98.5
lac04		*Lactobacillus*	*Lactobacillus agilis* DSM 20509^T	98.5
lac11	lac28	*Lactobacillus*	*Lactobacillus agilis* DSM 20509^T	98.0
lac14	lac07/lac08/lac17	*Lactobacillus*	*Lactobacillus agilis* DSM 20509^T	98.5
lac15		*Olsenella*	*Olsenella profusa* D315A-29^T	97.2
lac16		*Olsenella*	*Olsenella profusa* D315A-29^T	97.2
lac31^T		*Olsenella*	*Olsenella profusa* D315A-29^T	97.2
lac27		*Enterococcus*	*Enterococcus faecalis* JCM 20313	100
lac09	lac05/lac06	*Enterococcus*	*Enterococcus gallinarum* LMG 13129^T	99.8
lac12	lac29	*Enterococcus*	*Enterococcus gallinarum* LMG 13129^T	100
lac32		*Enterococcus*	*Enterococcus faecalis* JCM 20313	100
lac18^T		*Veillonella*	*Veillonella ratti* ATCC 17746^T	96.6

In total, 20 strains, the majority of the 27 isolates, were identified as belonging to the LAB *sensu stricto* (eight strains of the genus *Lactobacillus* and seven strains of the genus *Enterococcus*), *sensu lato* [one strain of the '*Bacillus cereus* group' (Kolstø *et al.,* 2009; Logan & De Vos, 2009) and three strains of the genus *Olsenella*] or the group of lactic acid-fermenting bacteria (one strain of the genus *Veillonella*). The remaining isolates were very closely related to *Escherichia coli* (seven strains). Given the relatively short length of most determined sequences, the percent sequence similarities of four strains (all strains of the genera *Olsenella* and *Veillonella*) to the most closely related strains of recognised species were clearly below the currently proposed cut-off level for species delineation (98.7-99 %; Stackebrandt & Ebers, 2006). A fifth strain, lac03, exhibited only 96.2 % sequence similarity to the type strain of *Lactobacillus delbrueckii* subsp. *indicus*, which was the most closely related recognised species at the time of isolation (data not shown). Later on, this strain was found to be more closely related (similarity of 98.5 %) and phenotypically similar to the independently and concurrently (Morita *et al.*, 2010) isolated strain *L. equicursoris* DSM 19284^T =DI70^T (Table 2). It therefore presumably belongs to the novel species *L. equicursoris* Morita *et al.* 2010. The phylogenetic UPGMA tree that was inferred using MEGA4 is shown in Fig. 1.

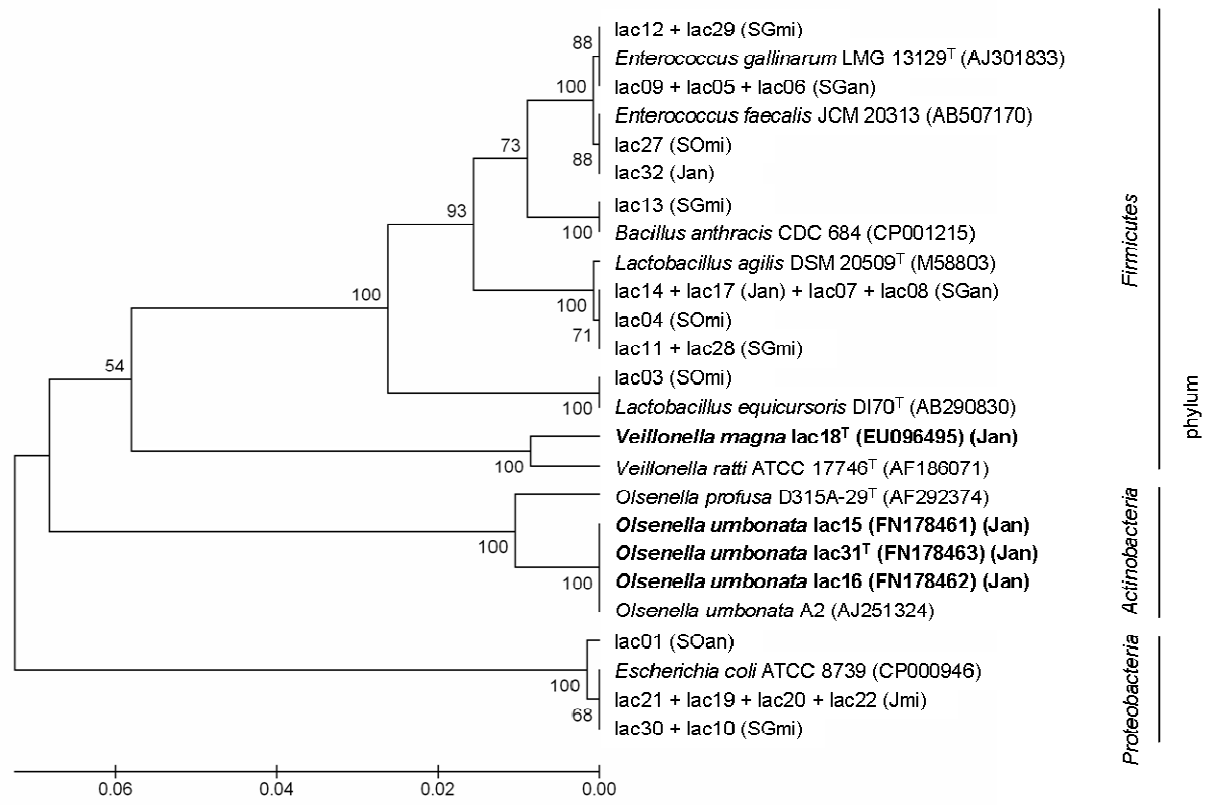

Fig. 1. **UPGMA tree of representative partial 16S rRNA gene sequences showing the phylogenetic relationships of mucosal isolates (strains of novel species in bold) and most closely related recognised strains. Accession numbers and origins of the newly isolated strains are given in parentheses (SO, SG, J, isolated from oesophageal stomach, glandular stomach, jejunum, respectively; an, mi, isolated under anaerobic, microaerobic conditions, respectively). Numbers at branch points are bootstrap values (%) based on 1000 replicates. Scale bar, 0-6 % sequence divergence.**

The UPGMA tree illustrated that the mucosal isolates belonged mainly to the *Firmicutes* of the *Bacteria*. This phylum included *Bacillus* strain lac13 and all strains of the LAB *sensu stricto* (enterococci and lactobacilli) of the class *Bacilli* as well as the lactic acid-fermenting strain lac18^T of the genus *Veillonella* of the newly described class *Negativicutes* (Marchandin *et al.,* 2010). The three isolates of the genus *Olsenella* (lac15, lac16 and lac31^T) represented the LAB *sensu lato* of the *Actinobacteria*. They formed a genetically coherent group (100 % inter-strain sequence similarities) with strain A2 from the rumen of a sheep (Eschenlauer *et al.,* 2002). This strain had been informally described as representative of '*Olsenella* (basonym *Atopobium*) *oviles*' (Dewhirst *et al.,* 2001; Eschenlauer *et al.,* 2002). Strain lac18^T and the four strains of the *Olsenella* group represented distinct taxa with respect to their closest phylogenetic neighbours, namely the species *Veillonella ratti* and *Olsenella profusa*. The genetic distinctness was in both cases strongly supported by a bootstrap value of 100 %.

Correlation between taxonomy, isolation source and atmospheric condition

The majority, i.e. seven of eight, of the facultatively anaerobic strains of the genera *Escherichia* and *Bacillus* were isolated under microaerobic conditions. The *Lactobacillus* and *Enterococcus* strains were isolated chiefly from the stomach and under both anaerobic and microaerobic conditions. Accordingly, these isolates were markedly microaerotolerant (5-7 % O_2) or aerotolerant anaerobes (Tables 1 and 2). Recovered from jejunal samples under anaerobic conditions, the strains of *O. umbonata* and *V. magna* were characterised as being less microaerotolerant (moderately obligate) anaerobes (Chapters V and VI). The strain *V. magna* lac18^T was isolated from a co-culture with lac32, the only *Enterococcus* strain from anaerobically incubated jejunum samples.

Discussion

Commercial PGM contains a mixture of secretory gel-forming mucins with predominantly *α*-*N*-acetylgalactosamine, *α*-fucose and unlike human gastric mucin also *α*-*N*-acetylglucosamine at the terminal ends of the oligosaccharide side chains (Hoskins *et al.,* 1985; Hoskins, 1992). It can be assumed that in this study, during the initial step of enrichment, an even more various mixture of mucins was present, in so far as the secreted gel-forming mucins, minor amounts of non-gel-forming (salivary and bronchial) mucins as well as cell-surface mucins (Linden *et al.,* 2008) from the mucosal samples were added to PGM.

Effectively exploitative growth on mucin by a bacterial community requires the presence of strains with active glycosulphatases, sialidases as well as *α*-exoglycosidases, such as *α*-fucosidase and *α*-*N*-acetyl-glucosaminidase, for the initial cleavage of sealing sulphate ester and sialic acid residues as well as terminal *α*-linked monosaccharides from the carbohydrate side chains. The used PGM contains 0.5-1.5 % bound sialic acids (Sigma-Aldrich, 2010). No active *α*-fucosidase was detected in the VITEK ANI analysis. This indicates that the isolated community was in fact more diverse and originally comprised some other functionally relevant members, possibly some deselected non-LARB with mucin oligosaccharide degrading capabilities. However, the *in vitro* analyses of mucinolytic glycosidases should be interpreted with caution, as bacterial enzymatic activities on the nitrophenylated monosaccharide derivatives in test kits may deviate from the activities on monosaccharides in the oligosaccharide chains of native mucins (Hoskins, 1992) and also, with regard to this study, as preformed enzymes from strains grown on rich BHG medium were determined.

The passage on PGM medium (PGM-plus without PYG) during the late steps of isolation ensured that the obtained isolates are capable of growth on mucin. Regarding this ability and the phenotypic characteristics mucinolytic glycosidases, mesophily and oxygen tolerance, the isolated bacterial community was principally adapted to the micro-oxic conditions in the mucosal microhabitats of the pig proximal gut (Isolauri *et al.,* 2004; Wilson, 2005).

Bacterial growth rates and population densities on mucins as substrate are comparatively low (Deplancke *et al.,* 2002) and, according to the nutrient-niche hypothesis, significantly enhanced by the interspecies cooperations in diverse communities (Bradshaw *et al.*, 1994; Laux *et al.,* 2005). In the present study, this was evident insofar as single colonies on the mucin-based agar plates were weak and highly uniform among the isolated strains. During the initial step of enrichment in liquid PGM-plus, the ecological stabiliser function of mucins (Hoskins *et al.,* 1985) probably prevented the overgrowth of the strains of *O. umbonata* and *V. magna*, which exhibited no mucinolytic glycosidases and are relatively fastidious (*O. umbonata*) or metabolically dependent (*V. magna*), by other potentially fast-growing bacteria. A long initial incubation time during enrichment is a prerequisite for the catabolite induction of mucin fermentation in the presence of high mucin and low glucose concentrations (Deplancke *et al.,* 2002; Macfarlane *et al.,* 2005) and for subsequent accumulation of lactic acid as substrate for, *inter alia*, veillonellae.

According to the results from 16S rRNA gene sequence-based phylogenetic analysis, the isolated strains of lactobacilli (n=8) and enterococci (n=7) belonged to only two different species of each genus. This low diversity of the LAB *sensu stricto* might be ascribed to the low pH of the PGM media. Growth of some lactobacilli is retarded at pH 5.2 (Sharpe, 1960), the pH measured in the uninoculated PGM-plus medium post autoclaving and addition of vitamin solution. It might also be attributed to allogenic and autogenic factors of the pig GI tract and the bacteria themselves. Firstly, the mucus layers of the pig stomach and proximal small intestine are inhospitable microhabitats due to a host of physicochemical determinants (swift peristalsis, high redox potential, acid and bile secretions, digestive enzymes, lysozyme and diverse other antimicrobial molecular secretions, direct antimicrobial activity of mucins) (Hao & Lee, 2004; Laux *et al.,* 2005; Pearson & Brownlee, 2005; Tannock, 2005; Linden *et al.,* 2008). Secondly, there is known competition (for adhesion) and antagonism (by antibacterial bacteriocins) within and between communities of lactobacilli and enterococci (Heinemann *et al.,* 2000; Ouwehand & Vesterlund, 2004; Parada *et al.,* 2007). Hence accordingly, the recurrent detection of *L. agilis* and *Enterococcus faecalis* from gastric and jejunal samples likely is due to proximal selection of strains, such as lac04 and lac27, in a ‘special foregut association’ (Walter, 2008) with the non-glandular epithelium of the stomach

pars oesophagea, shedding of cells of these strains from there and subsequent inoculation of digesta and distal mucosal microhabitats (Fuller *et al.,* 1978; Tannock, 2005).

PGM-plus and PGM are elective culture media that were designed for habitat-simulating approaches for isolation of mucosa-associated LARB. The only factor for selection against Gram-stain-negative enterobacteria was a low pH of 5.0. The new media did not contain any artificial selective ingredients such as high acetate or antibiotics. Therefore, and favoured by the fact that enterobacteria are numerically abundant in the microbiota of younger pigs (Leser *et al.,* 2002; Richards *et al.,* 2005; Tannock, 2005), six or one of 14 or 13 isolated strains were identified as *E. coli* using microaerobic or anaerobic culture conditions, respectively. This points out that only under microaerobic conditions, and despite a presumably concomitant election of more antagonistic lactobacilli (Annuk *et al.,* 2003), the facultatively anaerobic strains of *E. coli* had a significant advantage over the aerotolerant or microaerotolerant anaerobic LARB.

Overall, commercial PGM proved to be an adequate basal component in the enrichment isolation of complex communities of LARB, including strains of three novel species of phylogenetically diverse genera *Lactobacillus*, *Olsenella* and *Veillonella*, from the mucosae of the pig stomach and jejunum. Using anaerobic rather than microaerobic culture conditions, PGM-plus and PGM are sufficiently applicable and specific for LARB.

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Chapter V

Olsenella umbonata sp. nov., a microaerotolerant anaerobic lactic acid bacterium from the sheep rumen and pig jejunum, and emended descriptions of *Olsenella*, *Olsenella uli* and *Olsenella profusa*

Kraatz, M., Wallace, R. J. & Svensson, L. (in press). *Olsenella umbonata* sp. nov., a microaerotolerant anaerobic lactic acid bacterium from the sheep rumen and pig jejunum, and emended descriptions of *Olsenella*, *Olsenella uli* and *Olsenella profusa*. International Journal of Systematic and Evolutionary Microbiology. Published online April 30, 2010.

Strain A2 is an anaerobic, variably Gram-stain-positive, non-spore-forming, small and irregularly rod-shaped bacterium from the ruminal fluid of a sheep that has been informally described as representative of '*Olsenella* (basonym *Atopobium*) *oviles*'. Three phenotypically similar bacterial strains (lac15, lac16 and lac31^T) were isolated in concert with *Veillonella* magna lac18^T from the mucosal jejunum of a pig. A phylogenetic analysis based on 16S rRNA gene sequences revealed that the strains A2, lac15, lac16 and lac31^T formed a genetically coherent group (100 % inter-strain sequence similarities) within the *Olsenella-Atopobium* bigeneric branch of the family *Coriobacteriaceae*, class *Actinobacteria*. This group was most closely related to the two recognized *Olsenella* species, namely *Olsenella uli* (sequence similarity of 96.85 %) and *Olsenella profusa* (sequence similarity of 97.20 %). The sequence similarity to *Atopobium minutum*, the type species of the genus *Atopobium*, was 92.33 %. Unlike *Olsenella uli* and *Olsenella profusa*, outgrown colonies of the strains A2, lac15, lac16 and lac31^T displayed opaque, greyish-white umbonate elevations on solid culture media. The four novel strains were characterised as being well adapted and presumably indigenous to the gastrointestinal tract of homeothermic vertebrates: They were mesophilic, microaerotolerant, neutrophilic and acidotolerant, bile resistant, mucin-utilizing and markedly peptidolytic lactic acid bacteria. The results of DNA-DNA hybridizations, cellular fatty acid analysis and other differential phenotypic (physiological and biochemical) tests confirmed that the strains A2, lac15, lac16 and lac31^T represent a novel species of the genus *Olsenella*. On the basis of the genotypic and phenotypic results, we therefore formally describe *Olsenella umbonata* sp. nov., with lac31^T (=CCUG 58604^T =DSM 22620^T =JCM 16156^T) as the type strain and A2 (=CCUG 58212 =DSM 22619 =JCM 16157) as an additionally available strain. Also, based on our data, we propose emendations to the descriptions of the genus *Olsenella* and the species *Olsenella uli* and *Olsenella profusa*.

The genera *Olsenella* Dewhirst *et al.* 2001, *Atopobium* Collins and Wallbanks 1993 and *Bifidobacterium* Orla-Jensen 1924 constitute the high-G+C-content group of the lactic acid bacteria *sensu lato* (Inês *et al.,* 2008). The bacteria in this group are defined as members of the class *Actinobacteria* meeting the phenotypic core-criteria of Orla-Jensen (1919): They are Gram-stain-positive, non-motile, non-spore-forming rods or cocci that ferment carbohydrates to predominant (*Olsenella*, *Atopobium*) or important (*Bifidobacterium*) amounts of lactic acid.

The genus *Olsenella* comprises at present two species with validly published names: *Olsenella uli* Dewhirst *et al.* 2001 (basonym *Lactobacillus uli* Olsen *et al.* 1991) and *Olsenella profusa* Dewhirst *et al.* 2001 (prior designated *Eubacterium* group D52 by W. E. C. Moore and L. V. H. Moore). These species were based on strains from gingival and subgingival sites in humans with periodontitis. A third informal species with one strain (A2) from the rumen of a sheep had been named '*Atopobium oviles*' (Eschenlauer *et al.,* 2002) and was renamed '*Olsenella oviles*' (Dewhirst *et al.,* 2001).

According to Dewhirst *et al.* (2001), the human oral cavity is the main and the bovine rumen a likely habitat of the olsenellae. Isolates of *O. uli* and *O. profusa* are regularly recovered from disease sites in the human mouth (Munson *et al.,* 2002; Munson *et al.,* 2004; Hooper *et al.,* 2006) and sometimes from blood of humans with local oral or gastrointestinal infections (Lau *et al.,* 2004; Bahrani-Mougeot *et al.,* 2008). The olsenellae are found in the healthy (Ozutsumi *et al.,* 2005; Cho *et al.,* 2006; Hernandez *et al.,* 2008) and acidotic (Tajima *et al.,* 2000) bovine rumen. Molecular genetic studies also report the detection of *Olsenella*-related clones in the gastrointestinal tract of humans (Martinez-Medina *et al.,* 2006; Khachatryan *et al.,* 2008; Krogius-Kurikka *et al.,* 2009), pigs (Leser *et al.,* 2002; Tsukahara & Ushida, 2002; Dowd *et al.,* 2008), wallabies (Chhour *et al.,* 2008), chickens (Lu *et al.,* 2003) and in diverse anaerobic environmental sites (Bowman *et al.,* 2006; Wongtanet *et al.,* 2007; Weiss *et al.,* 2008; Rivière *et al.,* 2009). This indicates that the habitats of the olsenellae generally comprise the oral cavity and gastrointestinal tract of homeothermic vertebrates and that non-animal habitats coexist.

Strain A2 was isolated from the ruminal fluid of a sheep at the Rowett Institute of Nutrition and Health in 1994. The methods and results of the isolation, 16S rRNA gene sequence-based identification and initial phenotypic characterisation have been described in detail by Eschenlauer *et al.* (2002). The strains lac15, lac16 and lac31^T were isolated from the mucosal jejunum of a pig that also harboured lac18^T, the type strain of *Veillonella magna*, at the Institute of Animal Nutrition in 2007. The methods of the isolation and initial 16S rRNA gene sequence-based phylogenetic analysis using the primers 27f and 1492r have been described by Kraatz & Taras (2008).

Further genotypic studies included PCR amplification and direct sequencing of the 16S rRNA gene using the *Coriobacteriaceae* suited primer pair C75 and C90 (Dewhirst *et al.,* 2001). DNA was extracted from cells with a NucleoSpin Tissue kit (Macherey-Nagel). PCR was performed in a T1 Thermocycler (Biometra) using a HotStarTaq Master Mix kit (Qiagen). PCR products were purified with a High Pure PCR Product Purification kit (Roche). Sequencing was performed commercially by primer-walking (Eurofins MWG Operon) with C75 and C90 as starting primers. Near full-length (1428–1434 bp) 16S rRNA gene sequences of the four novel strains and strains of *O. uli* (*O. uli* DSM 7084^T), *O. profusa* (*O. profusa* DSM 13989^T, *O. profusa* CCUG 45371^T, *O. profusa* CCUG 45372) and *Atopobium minutum* (*A. minutum* DSM 20586^T) were obtained and submitted to EMBL (accession no. AJ251324 and FN178461-FN178468). Phylogenetic sequence analysis was conducted using the online Ribosomal Database Project II classification algorithm (Wang *et al.,* 2007; Cole *et al.,* 2009), the online NCBI BLASTn algorithm (Johnson *et al.,* 2008), MEGA software version 4.1

(Tamura *et al.,* 2007) and Consense from PHYLIP software package version 3.69 (Felsenstein, 2010). In MEGA4, alignments of the newly determined sequences and 14 related GenBank retrieved sequences were carried out using the CLUSTAL W tool (Thompson *et al.,* 1994). Pairwise evolutionary distances were computed by using the maximum composite likelihood method (Tamura *et al.,* 2004) with the Tamura-Nei nucleotide substitution model (Tamura & Nei, 1993) after complete deletion of positions with gaps or missing data. Phylogenetic trees were inferred using maximum-parsimony (MP) (Fitch, 1971), neighbour-joining (NJ) (Saitou & Nei, 1987) and minimum-evolution (ME) (Rzhetsky & Nei, 1992) methods. The bootstrap method (Felsenstein, 1985) was always used with 1000 replicates to test the statistical reliability of the trees. Genomic DNA-DNA reassociation analysis was carried out using hybridization protocols described by Urdiain *et al.* (2008). Labelled reference DNA of strains CCUG 58604^T or *O. profusa* CCUG 45371^T was hybridized to the unlabelled target DNA of strains CCUG 58212, *O. profusa* CCUG 45372 and *O. uli* CCUG 31166^T (as well as to homologous unlabelled DNA of CCUG 58604^T or CCUG 45371^T). Each hybridization mixture contained 150 ng of reference DNA and 15 µg of target DNA in a total volume of 72 µl. The mixtures were incubated for 16 h at 71 °C [i.e. at 30 °C below the melting point temperature of DNA with a G+C-content of, according to Dewhirst *et al.* (2001), 63-64 mol %].

The results of the 16S rRNA gene sequence-based phylogenetic analysis revealed that the strains lac15, lac16, lac31^T and A2 belonged to the genus *Olsenella* and formed a genetically coherent group (100 % inter-strain sequence similarities) within the *Olsenella-Atopobium* bigeneric branch of the family *Coriobacteriaceae*, class *Actinobacteria* (Fig. 1). *O. uli* and *O. profusa* were the most closely related recognized species (sequence similarities of 96.85 % and 97.20 %, respectively), and *A. minutum*, the type species of the genus *Atopobium*, was a more distant relative (92.33 %). The sequence similarity with respect to *Coriobacterium glomerans*, the type species of the *Coriobacteriaceae*, was 88.30-88.31 %. The genetic distinctness of the novel group within the *Olsenella-Atopobium* bigeneric branch was supported by bootstrap values of 88-92 % (mean, 90 %; *n*=10) in the MP consensus trees (Fig. 1). Bootstrap values were lower in the NJ and ME analyses [49-51 % and 51-53 % (mean, 50 % and 52 %; *n*=10), respectively], yet confirming the branching pattern of the MP trees (Supplementary Fig. S1). The results of the DNA-DNA hybridizations are presented in Supplementary Table S1. Relative reassociation of DNA of strain CCUG 58604^T was maximal (102.5 %) with DNA of strain CCUG 58212 and in the range of 50 % with respect to DNA of strains of *O. uli* (47.3 %) and *O. profusa* (50.2 and 51.9 %). DNA of strain *O. profusa* CCUG 45371^T exhibited even less than 50 % relative reassociation with DNA of strains CCUG 58604^T and CCUG 58212 (32.7 and 40.0 %, respectively). Altogether, the DNA-DNA relatedness between the novel strains CCUG 58604^T and CCUG 58212 and the

recognized strains of *O. uli* and *O. profusa* was clearly below the 70 % cut-off value for species delineation (Wayne *et al.,* 1987; Stackebrandt *et al.,* 2002; Achtman & Wagner, 2008). As indicated by the results of the 16S rRNA gene sequence-based phylogenetic analysis, the novel strains were related very closely to each other and more closely to *O. profusa* than to *O. uli*.

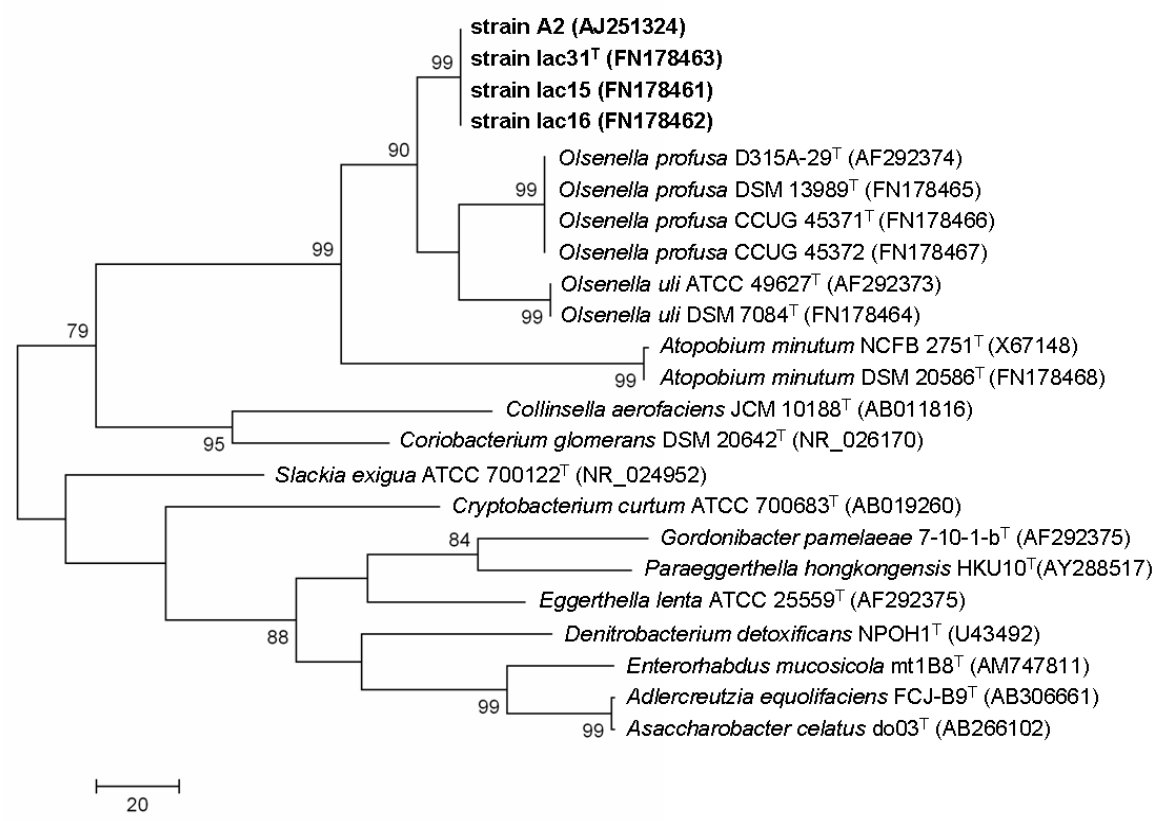
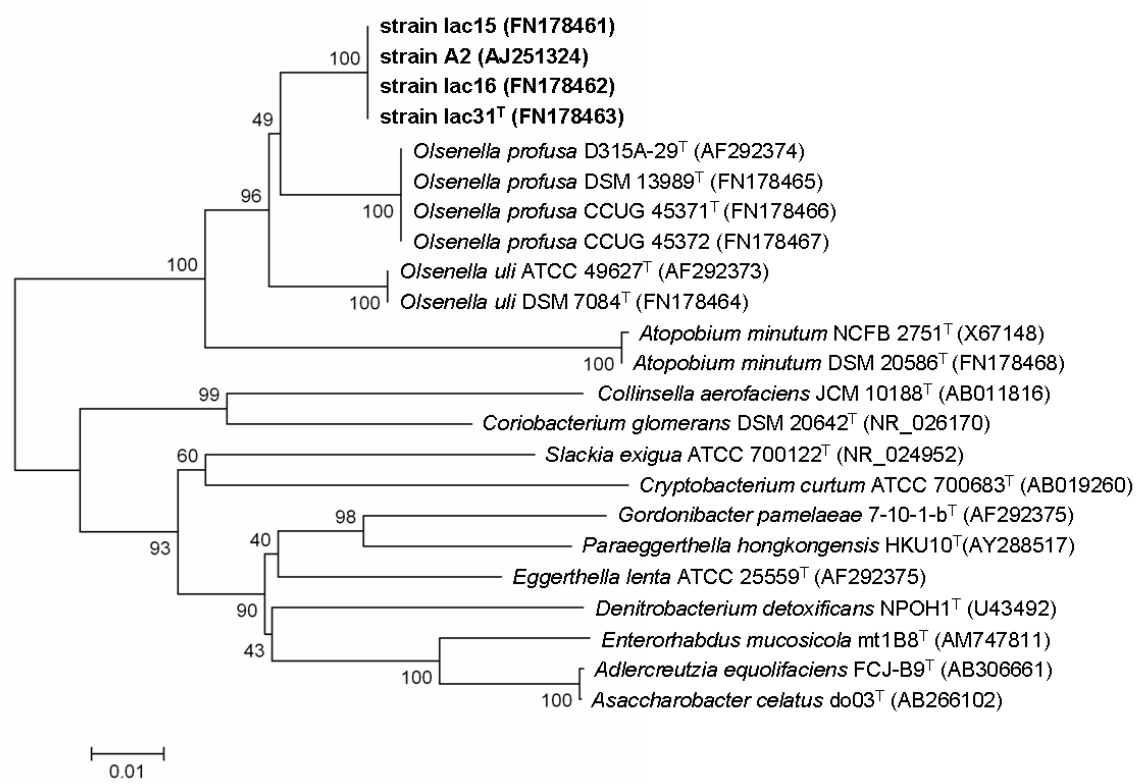


Fig. 1. **Maximum-parsimony tree of partial 16S rRNA gene sequences showing the phylogenetic relationship of the strains lac15, lac16, lac31^T, A2 (in bold) to other members of the *Olsenella-Atopobium* bigeneric branch and the family *Coriobacteriaceae*, including all type species. The tree is the unrooted consensus of 1000 bootstrap replicates. Bootstrap values (%) greater than 70 are given at branch points. Bar, 20 base changes between nodes. Accession numbers are given in parentheses.**



Supplementary Figure S1. **Neighbour-joining tree of partial 16S rRNA gene sequences showing the phylogenetic relationship of the strains lac15, lac16, lac31^T, A2 (in bold) to other members of the *Olsenella-Atopobium* bigeneric branch and the family *Coriobacteriaceae*, including all type species. The tree is the unrooted consensus of 1000 bootstrap replicates. Bootstrap values (%) are given at branch points. Bar, 0.01 estimated nucleotide substitutions per site. Accession numbers are given in parentheses.**

Supplementary Table S1. **Levels of DNA-DNA relatedness (%) as determined by hybridization of DNA of strains CCUG 58604^T and *Olsenella profusa* CCUG 45371^T to their homologous DNA and DNA of strains CCUG 58212, *O. profusa* CCUG 45372 and *O. uli* CCUG 31166^T. Values are means±standard deviations from two independent treatments [elution and detection of eluted DNA of each single hybridization mixture according to Urdiain *et al.* (2008)].**

	strain CCUG 58604^T	***O. profusa* CCUG 45371^T**
strain CCUG 58604^T	100.0±13.1	32.7±0.0
strain CCUG 58212	102.5±9.7	40.0±0.0
O. profusa CCUG 45371^T	50.2±5.8	100.0±4.7
O. profusa CCUG 45372	51.9±2.2	73.8±2.0
O. uli CCUG 31166^T	47.3±0.3	33.1±0.4

Phenotypic studies were performed using the following culture media: modified peptone-yeast extract-glucose (PYG) medium (medium DSMZ 104 including salt solution 104; http://www.dsmz.de) as the standard, unreduced PYG broth (medium DSMZ 104 without cysteine-HCl), peptone-yeast extract (PY) broth (medium DSMZ 104 without glucose), M2 liquid medium containing clarified rumen fluid (Hobson, 1969), fastidious anaerobe agar (FAA) (FAA plus 5 % defibrinated horse blood from Lab M and Oxoid, respectively), blood agar (blood agar base no. 2 plus 5 % defibrinated horse blood from Lab M and Oxoid, respectively), an unsupportive basal medium (UBM), porcine gastric mucin (PGM) agar, porcine gastric mucus-mucosa (PGMM) agar and porcine jejunal mucus-mucosa (PJMM) agar containing in the UBM 1 % (w/v) mucin from porcine stomach (type III; Sigma) (PGM) or mucus and mucosa from the stomachs (PGMM) or jejuna (PJMM) of healthy pigs. Unless otherwise stated, cultivations were performed at 37 °C. Anaerobic [i.e. anoxic, CO_2-enriched (18 % by vol.)], microaerobic [i.e. oxygen-reduced (5-7 % by vol.), CO_2-enriched (8-10 % by vol.)] and aerobic cultivations on agar media were performed in anaerobic jars using Anaerocult A gas packs, Anaerocult C gas packs (both from Merck) and no gas packs, respectively. Anaerobic and microaerobic cultivations in broth media were performed in anaerobically sterilised (nitrogen-purged) and aerobically sterilised (unpurged), respectively, rubber-stoppered glass tubes (110 by 15 mm). Growth in broth media was monitored by measuring optical density (OD) at 600 nm with an Ultrospec 2000 spectrophotometer (Pharmacia Biotech). Studies were usually carried out with three or five (analyses using API kits) repetitions for each strain.

Morphology was studied using light and scanning electron microscopy (SEM) with cells grown anaerobically on FAA and PYG agar for 48 h and 7 d and on PGM, PGMM and PJMM agar for 14 d. The cell size was determined using the measurement tool of the Photoshop CS4 Extended software (Adobe) on digital SEM photographs. The morphological studies and the studies on motility, spore formation, cytochrome oxidase activity and nitrate reduction were conducted as described previously (Kraatz & Taras, 2008). The oxygen relationship was determined using anaerobic cultures on PYG agar and in PYG broth, microaerobic cultures on PYG agar and in unreduced aerobically sterilised (URAS) PYG broth and aerobic cultures on PYG agar. Oxygen levels in the culture media were assessed by adding 1 mg resazurin sodium salt (Sigma) l^{-1} as redox indicator (orange or red indicative colour of the reduced or oxidized form of resazurin in anaerobic or microaerobic and aerobic PYG medium). Referring to the approach of Karakashev *et al.* (2003), assessment of growth and changed redox status in microaerobic URAS liquid media with resazurin allows further differentiation of the obligately anaerobic bacteria (Winn *et al.,* 2006) into the microaerotolerant (moderately obligate) anaerobes and the strictly obligate anaerobes. The URAS PYG broth cultures of the novel strains and strains of *O. uli* and *O. profusa* were

prepared as 7.5-ml volumes with an initial OD of 0.03-0.18 (mean, 0.09; *n*=24) above that of the uninoculated controls. Analyses of the range for growth at different temperatures (21, 30, 37, 45 °C) and pH values (initial pH value 2.0-9.5 at intervals of 0.5 pH units), growth resistance tests with 20 % bile (Jousimies-Somer *et al.,* 2002) or 6.5 % (w/v) NaCl and growth stimulation tests with 0.1 % (v/v) Tween 80 or 0.5 % (w/v) L-arginine were carried out using anaerobic cultures in PYG broth. Production of hydrogen sulfide from meat peptone or L-cysteine was studied using anaerobic cultures in UBM broth enriched with trypticase peptone and glucose and supplemented with 0.5 g ferric ammonium citrate l^{-1} and 10 g meat peptone or 0.73 g L-cysteine l^{-1}. Production of gas from 1 % (w/v) glucose was examined using anaerobic stab cultures in PYG agar deeps following the method for anaerobes of Smibert & Krieg (1994). For the analysis of hydrogen peroxide production (Juárez Tomás *et al.,* 2004), cells were grown on a PYG agar without resazurin and supplemented with 0.01 g peroxidase from horseradish (type VI-A; Sigma) l^{-1} and 20 ml TMB (3,3′,5,5′-tetramethylbenzidine; Sigma) solution (25 mg TMB ml^{-1} ≥99.8 % ethanol) l^{-1}. The hydrogen peroxide assays were incubated anaerobically for 7 d, then exposed to air under light-protected conditions at room temperature for 24 h and evaluated as described by Otero & Nader-Macías (2006). Biochemical and enzyme profiles were determined with the API 20 A, Rapid ID 32 A and API ZYM kits (bioMérieux) according to the manufacturer's instructions. The inocula were prepared using anaerobic 24 h-old (API 20 A) or, in accordance with Dewhirst *et al.* (2001), 72 h-old (Rapid ID 32 A and API ZYM) cultures on blood agar. The API 20 A tests were evaluated after 48 h of incubation. Growth on and fermentation of carbohydrates not included or with ambiguous result in the API 20 A were analysed using anaerobic cultures in PY broth supplemented with 1 % (w/v) inulin or 20 mM D-raffinose and in UBM broth enriched with trypticase peptone and supplemented with 20 mM D-fructose, D-xylose, α-L-rhamnose, respectively, or 10 mM D-melibiose. Production of short-chain volatile and non-volatile fatty acids and ammonium was analysed using anaerobic cultures in PYG broth and microaerobic cultures in URAS PYG broth. Meat extract was omitted for the analysis of lactic acids. Volatile fatty acids were determined using GC analysis as described previously (Schäfer, 1995). Non-volatile fatty acids were determined using HPLC analysis and (D- and L-lactic acid)/or (succinic acid) enzymatic UV-tests. HPLC analysis was carried out on a HP 1100 Series system (Agilent Technologies) equipped with a 150 x 4.6 mm analytical column coated with N,S-dioctyl-D-penicillamine and a 4.0 x 2.0 mm SecurityGuard C18 guard column (both from Phenomenex). The columns were operated at 35 °C. The eluents were solutions of $CuSO_4$ in (A) water (0.5 mmol l^{-1}) and (B) water with 5 % isopropanol (2.5 mmol l^{-1}) in a gradient mode (0-35 % B over 17 min, 35 % B for 2 min, 35-100 % B over 3 min, 100 % B for 1 min, 100-35 % B over 1 min) and running with a flow rate of 1.0 ml min^{-1}. The total injection volume for analysis was 20 µl of 40 % (v/v) culture supernatant in eluent A. Compounds were UV-detected at 254 nm and identified by

comparison against retention times using a lithium-DL-lactate standard (AppliChem). Chromeleon chromatography software (Dionex) was used for quantification analysis. Enzymatic UV-tests (R-Biopharm) were applied according to the manufacturer's instructions and using an Ultrospec 3300 pro UV/visible spectrophotometer (Amersham Biosciences) at 365 nm. Ammonium was measured electrometrically using a DC218-NH4 ammonium electrode in combination with a SevenMulti S80 ion meter (both from Mettler Toledo). For the analysis of cellular fatty acids, bacteria were anaerobically grown overnight in 100 ml of M2 liquid medium. Saponification, fatty acid methylation and analysis of fatty acid methyl esters was carried out as described previously (Devillard *et al.,* 2006). Three independent cultures, each grown on the same batch of M2, were always analysed for each strain. For some strains, the analysis was repeated using triplicate cultures on another, different batch of the medium.

The results of the phenotypic characterisation are given in the species description. Phenotypic characteristics that serve to differentiate the strains A2, lac15, lac16 and lac31^T from the type and reference strains of *O. uli* and *O. profusa* are given in Tables 1 and 2 and Supplementary Table S2. On the basis of the results of 16S rRNA gene sequencing, DNA-DNA reassociation analyses and the differential phenotypic data, the strains A2 [formerly named '*Olsenella* (basonym *Atopobium*) *oviles*'], lac15, lac16 and lac31^T represent a novel species of the genus *Olsenella*, for which we propose the name *Olsenella umbonata*. Also, on the basis of the data from this study, we propose emendations to the descriptions of the genus *Olsenella* and the species *O. uli* and *O. profusa*.

Table 1. Differential phenotypic characteristics of strains lac15, lac16, lac31^T, A2 and strains of *O. uli* and *O. profusa*

Taxa: 1-4, lac15, lac16, lac31^T, A2; 5, O. uli DSM 7084^T; 6-8, O. profusa DSM 13989^T, O. profusa CCUG 45371^T, O. profusa CCUG 45372. +, positive; -, negative; w, weak. FAA, fastidious anaerobe; PYG, peptone-yeast extract-glucose; PGM, porcine gastric mucin; URAS, unreduced aerobically sterilised. Data were obtained in the present study.

Characteristic	**1-4**	**5**	**6-8**
Colony and cell morphology (on FAA and PYG agar, 7 d)			
Circular diameter (FAA/PYG; mm)	≤3-4/4	≤2/2.5	≤2/3
Elevation	umbonate	raised, central punctiform knob	pulvinate
Opacity (margin/centre)	semi-translucent/opaque	semi-translucent/opaque	opaque/opaque
Colour (margin/centre)	grey/greyish-white	grey/greyish-white	cream-white/cream-white
Texture	butyrous	butyrous	granular
Cell arrangement	single, pair, short or longer chain	single, pair, short or longer chain	short to very long serpentine chain
Gram-stain variability	marked	slight	slight
Microaerobic growth in URAS PYG broth	good	- to moderate	- to good
ODΔ4d-0h, min/mean/max(0h)*	1.43/1.65/1.79(0.09)	0.00/0.36/1.08(0.04)	0.00/1.29/1.56(0.04)
Accumulation of hydrogen peroxide	-	-	- or w
Growth on mucin	moderate to good	moderate	moderate
Colony diameter on PGM agar, 14 d (mm)	≤1-2	≤1	≤1
Major cellular fatty acid	14:0/18:0	18:0	*anteiso*-methyl-14:0

*Values are from 12 (taxa 1-4), 3 (taxon 5) and 9 (taxa 6-8) repetitions.

Table 2. Differential biochemical and enzyme characteristics of strains lac15, lac16, lac31^T, A2 and strains of *O. uli* and *O. profusa*

Taxa: 1-4, lac15, lac16, lac31^T, A2; 5, O. uli DSM 7084^T; 6-7, *O. profusa* DSM 13989^T, *O. profusa* CCUG 45371^T. +, positive; -, negative; v, variable. For a variable reaction, the reaction of the type strain is that of the majority of strains and is given in parentheses. In the Rapid ID 32 A, all strains were negative for the following: urease, *α*-galactosidase, *α*-arabinosidase, *β*-glucuronidase, raffinose fermentation, reduction of nitrates, indole production, pyroglutamic acid arylamidase, glutamic acid decarboxylase, *α*-fucosidase, glutamyl glutamic acid arylamidase. In the Rapid ID 32 A, all strains were positive for the following: arginine dihydrolase, mannose fermentation, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, serine arylamidase. In the API 20 A, all strains were negative for the following: indole formation, urease, acidification of glycerol and melezitose, catalase, spores, morphology. In the API 20 A, all strains were positive for the following: acidification of glucose, gelatin hydrolysis (protease), Gram reaction. In the API ZYM, all strains were negative for the following: trypsin, *α*-chymotrypsin, *α*-galactosidase, *β*-glucuronidase, *α*-mannosidase, *α*-fucosidase. Values for the API ZYM are means of the activity mark±standard deviations, *n*=20 (strains 1-4), *n*=5 (strains 5 and 6-7, respectively). The activity mark is a measure of the quantity of hydrolysed substrate (nmol): 0=<5; 1=5-<10; 2=10-<20; 3=20-<30; 4=30-<40; 5=≥40. Data were obtained in the present study unless indicated. API 20 A and API ZYM data have not been reported previously by Olsen *et al.* (1991) or Dewhirst *et al.* (2001).

Characteristic	**1-4**	**5**	**6-7**
Rapid ID 32 A profile	2402073705	2012073705*	2713473705*
β-Galactosidase	-	-	+
6-Phospho-*β*-galactosidase	-	-	+
α-Glucosidase	+	-	+
β-Glucosidase	-	+	+
N-acetyl-*β*-glucosaminidase	-	-	+
Alkaline phosphatase	-	-	+
API 20 A profile	441240(2/0)2	40060002	47776632
Esculin hydrolysis (*β*-glucosidase)	-	+	+
Acidification of:			
Mannitol	-	-	+
Lactose	-	-	+
Saccharose	+	-[a]†	+
Maltose	+	-[a]	+
Salicin	-	-[a]	+
Xylose	-	-	+‡
Arabinose	-	-	+
Cellobiose	-	-	+
Mannose	+	-[a]	+
Raffinose	-	-	+
Sorbitol	-	-	+‡
Rhamnose	-	-	+[b]
Trehalose	v (+)	-	+

Characteristic	1-4	5	6-7
Fermentation of melibiose§	-	-	+
Semi-quantitative enzyme activities (API ZYM)			
Alkaline phosphatase	0.7 ± 0.5	1.8 ± 0.4	2.4 ± 0.5
Esterase (C4)	2.3 ± 0.4	1.2 ± 0.4	1.4 ± 0.5
Esterase lipase (C8)	1.8 ± 0.4	1.2 ± 0.4	1.2 ± 0.4
Lipase (C14)	0.6 ± 0.5	0.0 ± 0.0	1.0 ± 0.0
Leucine arylamidase	3.7 ± 0.5	3.2 ± 0.4	3.4 ± 0.5
Valine arylamidase	1.9 ± 0.4	1.4 ± 0.5	3.2 ± 0.4
Cystine arylamidase	0.8 ± 0.4	0.6 ± 0.5	2.4 ± 0.5
Acid phosphatase	3.5 ± 0.7	3.6 ± 0.5	3.2 ± 0.4
Naphtol-AS-BI-phosphohydrolase	2.3 ± 0.4	2.2 ± 0.4	4.2 ± 0.4
β-Galactosidase	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.5
α-Glucosidase	4.2 ± 0.8	0.0 ± 0.0	1.0 ± 0.0
β-Glucosidase	0.0 ± 0.0	3.0 ± 0.0	0.0 ± 0.0
N-acetyl-*β*-glucosaminidase	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.4

*Profile reported by Dewhirst *et al.* (2001) is 2012033705 (1 reaction different from the present result) for *O. uli* and 4516053705 (7 reactions different from the present result) for *O. profusa*.

†Different from result reported by: a, Olsen *et al.* (1991); b, Dewhirst *et al.* (2001). In these studies, results were determined using the methods of Holdeman *et al.* (1977).

‡Not determined by Dewhirst *et al.* (2001).

§Data for *O. uli* and O. *profusa* taken from Olsen *et al.* (1991) and Dewhirst *et al.* (2001), respectively.

Supplementary Table S2. Cellular fatty acid composition of strains lac15, lac16, lac31^T, A2 and strains of *Olsenella uli* and *O. profusa*

Taxa: 1, lac15; 2, lac16; 3, lac31^T; 4, A2; 5, *O. uli* DSM 7084^T; 6, *O. profusa* DSM 13989^T; 7, *O. profusa* CCUG 45371^T; 8, *O. profusa* CCUG 45372. Values (%) are range amounts derived from three (taxa 2, 3, 6 and 8), six (taxa 1, 5 and 7) or nine (taxon 4) independent cultures in M2 liquid medium, cellular fatty acid extractions and analyses of fatty acid methyl esters. Ranges of total saturated (including unbranched and *anteiso*-branched forms) and unsaturated fatty acids are given in bold. Results using batches 1, 2, 1+3, 2+3 and 1+2+3 of M2 medium containing clarified rumen fluids are shaded in white, light grey, medium grey, dark grey and black, respectively. Data were obtained in the present study.

Fatty acid	**1**	**2**	**3**	**4**	**1-4**	**5**	**6**	**7**	**8**	**6-8**
Saturated										
Unbranched										
12:0	1.7-3.3	2.8-4.0	2.8-3.0	1.5-24.2*	1.5-24.2	1.1-2.8	0.1-0.1	0.1-0.3	0.2-0.3	0.1-0.3
13:0	0.0-0.6	0.4-0.6	0.5-0.6	0.0	0.0-0.6	0.0-1.1	0.9-1.2	0.7-1.2	1.8-2.0	0.7-2.0
14:0	31.6-43.4	25.3-32.8	24.6-26.7	60.9-89.4	24.6-89.4	3.4-4.6	10.6-11.9	12.5-14.9	18.8-19.6	10.6-19.6
16:0	4.3-14.7	2.1-8.2	6.4-11.3	3.5-12.0	2.1-14.7	10.3-18.1	1.3-1.5	3.0-10.5	1.5-1.8	1.3-10.5
18:0	26.1-52.7	53.9-58.7	51.0-58.8	2.0-12.8	2.0-58.8	31.7-50.5	0.2-0.3	0.0-9.6	0.4-0.6	0.0-9.6
(Total unbranched)	**(82.1-98.0)**	**(94.0-98.1)**	**(90.7-95.6)**	**(88.4-97.7)**	**(82.1-98.1)**	**(49.5-71.1)**	**(13.1-15.0)**	**(18.4-35.2)**	**(22.8-24.2)**	**(13.1-35.2)**
Anteiso-branched										
Anteiso-methyl-13:0	0.0-0.3	0.0	0.0	0.0-0.2	0.0-0.3	0.0-0.4	9.3-9.9	6.3-10.8	8.6-9.6	6.3-10.8
Anteiso-methyl-14:0	0.5-1.4	0.1-0.6	0.5-1.1	0.0-1.1	0.0-1.4	1.8-3.2	68.7-70.3	50.5-66.8	58.2-58.9	50.5-70.3
Total *anteiso*-branched	**0.5-1.7**	**0.1-0.6**	**0.5-1.1**	**0.0-1.3**	**0.0-1.7**	**2.2-3.2**	**78.5-79.6**	**56.9-76.1**	**66.8-68.3**	**56.9-79.6**
Iso-branched										
Iso-methyl-14:0	1.3-2.5	1.6-2.2	1.8-2.1	0.3-2.1	0.3-2.5	2.0-12.4	1.1-1.3	0.5-1.1	1.8-1.8	0.5-1.8
(Total branched)	(1.8-3.2)	(1.9-2.7)	(2.6-3.2)	(0.6-2.4)	(0.6-3.2)	(4.1-15.5)	(79.6-80.9)	(57.6-77.0)	(68.7-70.1)	(57.6-80.9)
Total saturated	**85.2-99.9**	**96.3-100**	**93.9-98.2**	**90.8-98.8**	**85.2-100**	**54.0-86.5**	**94.0-94.5**	**92.8-96.7**	**92.9-92.9**	**92.8-96.7**

Fatty acid	1	2	3	4	1-4	5	6	7	8	6-8
Monounsaturated										
15:1	0.0	0.0	0.0	0.0	0.0	0.0	3.9-4.9	1.7-3.8	5.6-5.9	1.7-5.9
16:1	0.8-1.2	0.6-0.7	0.8-0.8	0.3-0.9	0.3-1.2	1.8-4.0	0.0-0.6	0.0-0.2	0.0-0.2	0.0-0.6
18:1 *cis*-9	2.2-3.4	1.4-1.9	2.3-3.3	0.5-1.9	0.5-3.4	5.8-24.5	0.0-0.1	0.0-0.7	0.1-0.1	0.0-0.7
18:1 *trans*-9	2.4-5.2	4.8-6.2	4.0-5.0	0.0-1.1	0.0-6.2	0.0-3.4	0.0	0.0	0.0	0.0
18:1 *trans*-11	1.5-2.4	1.8-2.4	2.0-2.4	0.0-1.5	0.0-2.4	2.4-5.9	0.0	0.0-1.5	0.0-0.2	0.0-1.5
(Total monounsaturated)	(8.1-10.0)	(9.0-10.0)	(9.9-10.7)	(0.9-5.4)	(0.9-10.7)	(12.8-34.2)	(4.5-5.0)	(2.4-4.0)	(6.0-6.1)	(2.4-6.1)
Polyunsaturated										
18:2 *cis*-9, *cis*-12	0.0-1.1	0.0	0.0-0.9	0.0-1.2	0.0-1.2	5.6-18.4	0.0	0.0-0.8	0.0-0.4	0.0-0.8
Total unsaturated	**9.1-10.5**	**9.0-10.0**	**10.1-11.2**	**0.9-6.6**	**0.9-11.2**	**23.1-40.1**	**4.5-5.0**	**2.4-4.8**	**6.0-6.5**	**2.4-6.5**

*High amounts (22.8±0.9; *n*=3) were obtained using the original stock culture of A2 at the Rowett Institute of Nutrition and Health.

Emended description of the genus *Olsenella* Dewhirst *et al.* 2001

The description remains as given by Dewhirst *et al.* (2001) with the following emendations. Cells are consistently or variably Gram-stain-positive. They occur singly, in pairs and in short to very long serpentine chains. Cells are microaerotolerant (moderately obligate) anaerobic. Lactic acid is the major metabolic product from glucose. Minor products from glucose are formic and acetic acids. The cellular fatty acids consist mainly of saturated fatty acids. The major cellular fatty acid is variable, i.e. saturated unbranched or saturated branched. Found in the oral cavity and gastrointestinal tract of humans, other mammals and likely of homeothermic vertebrates.

Emended description of *Olsenella uli* (Olsen *et al.* 1991) Dewhirst *et al.* 2001

The description remains as given by Dewhirst *et al.* (2001) with the following emendations. Cells are microaerotolerant (moderately obligate) anaerobic (less than approx. 5 % O_2, by vol.). They are able to grow in microaerobic URAS PYG broth. The cellular fatty acids consist mainly of saturated unbranched fatty acids. The major cellular fatty acid of cells grown in M2 liquid medium is 18:0 (octadecanoic acid) (Supplementary Table S2, Table 1). Additional phenotypic characteristics of the type strain *O. uli* DSM 7084^T are given in Tables 1 and 2.

Emended description of *Olsenella profusa* Dewhirst *et al.* 2001

The description remains as given by Dewhirst *et al.* (2001) with the following emendations. Cells are microaerotolerant (moderately obligate) anaerobic (less than approx. 5 % O_2, by vol.). They are able to grow in microaerobic URAS PYG broth. The cellular fatty acids consist predominantly of saturated *anteiso*-branched fatty acids. The major cellular fatty acid of cells grown in M2 liquid medium is *anteiso*-methyl-14:0 (12-methyl-tetradecanoic acid) (Supplementary Table S2, Table 1). Additional phenotypic characteristics of the type strain *O. profusa* DSM 13989^T =CCUG 45371^T and the additional strain *O. profusa* CCUG 45372 are given in Tables 1 and 2.

Description of *Olsenella umbonata* sp. nov.

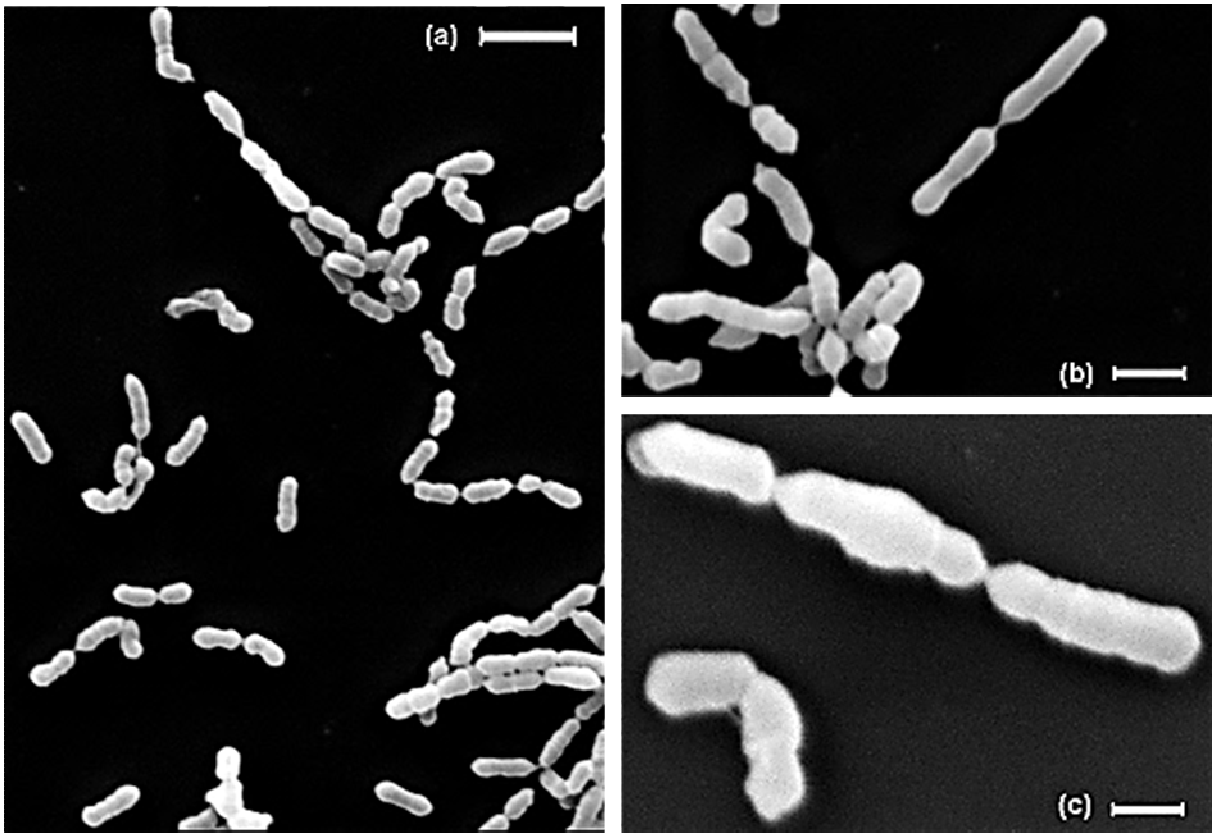
Olsenella umbonata [um.bo.na'ta. N.L. fem. adj. *umbonata* bossed, umbonate (from L. m. n. *umbo umbonis* shield boss) referring to the umbonate elevations of outgrown colonies on solid culture media].

Cells grown on FAA and PYG agar plates for 48 h under anaerobic conditions at 37 °C form colonies that are circular, up to 1.5 mm in diameter, have entire margins and smooth surfaces and are raised to slightly umbonate, semi-translucent, greyish-white and butyrous. Outgrown colonies on FAA and PYG agar display opaque, greyish-white umbonate elevations (Table 1).

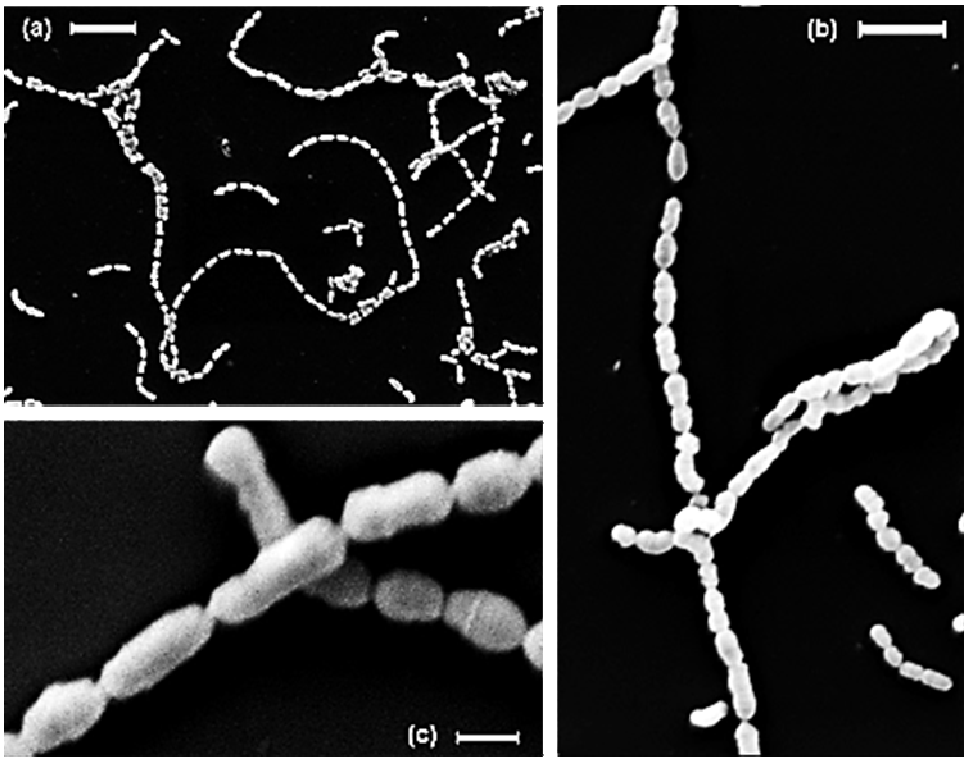
No haemolysis occurs on FAA or blood agar. After anaerobic incubation on PGM, PGMM and PJMM agar plates at 37 °C for 14 d, colonies are circular, punctiform (PGMM) to 1 mm (PJMM) or 2 mm (PGM) in diameter, have entire margins and smooth surfaces and are flat (PGMM, PJMM) to slightly umbonate (PGM), translucent and butyrous. Cells grown under anaerobic conditions on PYG agar for 48 h and on PGM agar for 14 d are small [0.3-0.6 µm (mean, approx. 0.4 µm; *n*=78 and *n*=46, respectively) × 0.6-2.2 µm (mean, approx. 1.1 µm; *n*=62 and *n*=209, respectively)], irregular (centrally or terminally swollen) and occasionally curved rods (Supplementary Fig. S2 and S3). Cells grown on PJMM agar are slightly less in length (0.5-2.0 µm; mean, approx. 1.0 µm; *n*=40) and thus more often appear coccoid (Supplementary Fig. S4). Cells are variously arranged (Table 1). Very long serpentine chains occur with cells long grown on PGM agar (Fig. S3). Cells are less (48 h-old cells) or more (7 d-old cells) variably Gram-stain-positive (Table 1). Cells are non-motile and non-spore-forming. Cytochrome oxidase activity is not detected. Nitrate is not reduced. Cells are microaerotolerant (moderately obligate) anaerobic (less than approx. 5 % O_2, by vol.). They routinely grow in microaerobic URAS PYG broth (Table 1). During growth, the resazurin in URAS PYG broth is reduced (colour change of the medium from red to orange). Cells are mesophilic; growth is absent at 21 °C, moderately good at 30 °C, very good at 37 °C and good at 45 °C. Cells are neutrophilic and acidotolerant; the pH range for growth is 4.5-8.0, occasionally 8.5 (optimum, pH 6.0-7.0). Growth is positive in 20 % bile and is absent in 6.5 % NaCl. Growth is markedly stimulated by 0.1 % Tween 80 and is unstimulated by 0.5 % L-arginine. Hydrogen sulfide is not produced from meat peptone or L-cysteine. No gas is detected in agar deeps. Hydrogen peroxide is not accumulated. Catalase activity is negative. Biochemical and enzyme characteristics using the API kits are listed in Table 2. In the API 20 A, acidification of trehalose is variable due to a negative reaction of the strain A2. Growth on and fermentation of D-fructose is positive. Growth on and fermentation of D-melibiose (Table 2), inulin, D-raffinose, *α*-L-rhamnose and D-xylose is negative. Cells are able to grow on mucin from porcine stomach (Table 1). Under anaerobic conditions, glucose is metabolised to

predominantly D-lactic acid (mean, approx. 39 mmol l^{-1}; *n*=12) and to minor amounts of formic acid (mean, approx. 4.5 mmol l^{-1}; *n*=12) and acetic acid (mean, approx. 3.3 mmol l^{-1}; *n*=12). Under unreduced microaerobic conditions, metabolisation of glucose is not decreased regarding the production of D-lactic acid (mean, approx. 39 mmol l^{-1}; *n*=10) and is slightly decreased regarding the production of formic acid and acetic acid [mean, approx. 2.5 mmol l^{-1} (*n*=12) and 2.2 mmol l^{-1} (*n*=12), respectively]. Glucose is not metabolised to L-lactic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, caproic acid and succinic acid. Presumably, cells are obligately homofermentative and produce formic and acetic acids via the anaerobic pyruvate-formate lyase system (Axelsson, 2004). Lactic acid is a reducing agent and contributes to the oxygen tolerance of cells (Brioukhanov & Netrusov, 2007). Ammonium is produced from peptone under anaerobic and unreduced microaerobic conditions [mean, approx. 12 mmol l^{-1} (*n*=12) and 9 mmol l^{-1} (*n*=12), respectively]. The cellular fatty acids consist predominantly of saturated unbranched fatty acids. The major cellular fatty acids of cells grown in M2 liquid medium are saturated, 14:0 (tetradecanoic acid) and 18:0 (octadecanoic acid) in varying proportions (Supplementary Table S2, Table 1). Well adapted and presumably indigenous to the gastrointestinal tract of homeothermic vertebrates, as suggested by the phenotypic characteristics mesophily, obligate anaerobiosis and microaerotolerance [in regard to the micro-oxic conditions at the absorptive mucosae (Isolauri *et al.,* 2004; Wilson, 2005)], neutrophily and acidotolerance, bile resistance, mucin utilization.

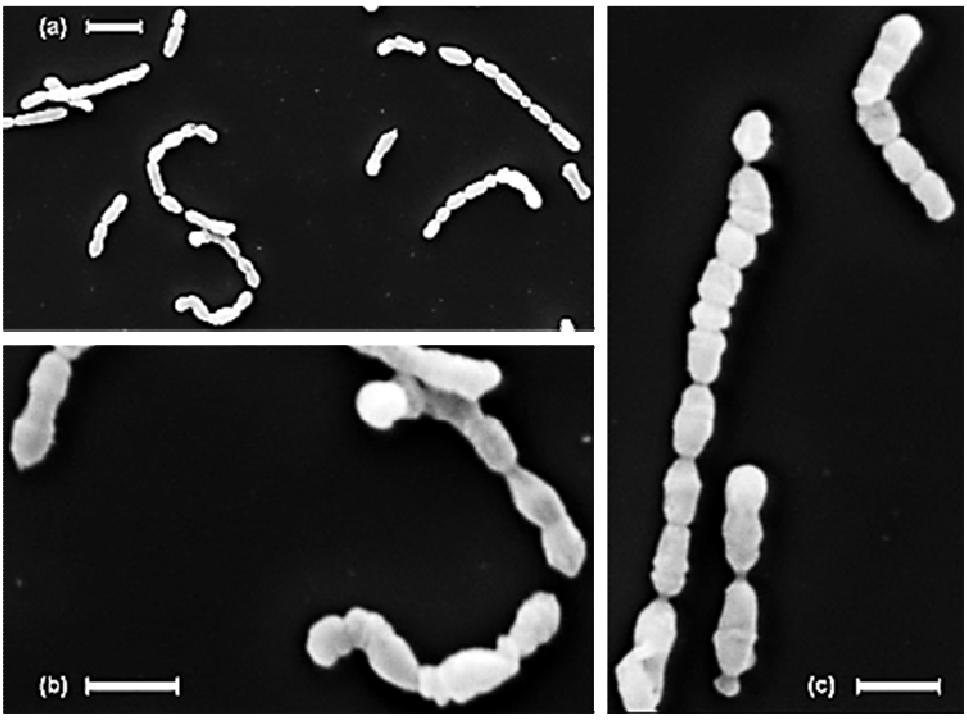
The type strain, lac31^T (=CCUG 58604^T =DSM 22620^T =JCM 16156^T), was isolated from the mucosal jejunum of a pig in Berlin, Germany. Strain A2 (=CCUG 58212 =DSM 22619 =JCM 16157) was isolated from the ruminal fluid of a sheep in Aberdeen, United Kingdom. It can be readily differentiated from the type strain by a negative acidification of trehalose in the API 20 A. Strains lac15 and lac16, isolated together with the type strain, are also included in the species.



Supplementary Figure S2. **Scanning electron micrographs of cells of strain lac31^T. Bar: (a) 2 µm; (b) 1 µm; (c) 0.5 µm. Cells were grown on peptone-yeast extract-glucose (PYG) agar under anaerobic conditions for 48 h at 37 °C.**



Supplementary Figure S3. **Scanning electron micrographs of cells of strain A2. Bar: (a) 5 µm; (b) 2 µm; (c) 0.5 µm. Cells were grown on porcine gastric mucin (PGM) agar under anaerobic conditions for 14 d at 37 °C.**



Supplementary Figure S4. **Scanning electron micrographs of cells of strain lac31^T. Bar: (a) 2 µm; (b), (c) 1 µm. Cells were grown on porcine jejunal mucus-mucosa (PJMM) agar under anaerobic conditions for 14 d at 37 °C.**

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Chapter VI

Veillonella magna sp. nov., isolated from the jejunal mucosa of a healthy pig, and emended description of *Veillonella ratti*

Kraatz, M. & Taras, D. (2008). *Veillonella magna* sp. nov., isolated from the jejunal mucosa of a healthy pig, and emended description of *Veillonella ratti*. International Journal of Systematic and Evolutionary Microbiology 58, 2755-2761.

A bacterium, designated strain lac18^T, was isolated in pure culture from the mucosal jejunum of a healthy pig, using a medium selective for anaerobic lactic acid bacteria and containing porcine gastric mucin as the main carbon and nitrogen source. Cells of this strain were coccus-shaped, arranged singly or in pairs and were Gram-stain-negative, oxidase-negative, non-spore-forming, anaerobic and microaerotolerant. An analysis based on 16S rRNA gene sequences indicated that strain lac18^T should be assigned to the genus *Veillonella*, class *Clostridia*, phylum *Firmicutes*. 16S rRNA and dnaK gene sequence-based phylogenetic analyses both indicated that the most closely related species were *Veillonella ratti* ATCC 17746^T (similarities of 96.6 % and 84.5 %, respectively) and *Veillonella criceti* ATCC 17747^T (similarities of 96.6 % and 83.4 %, respectively). The results of DNA-DNA hybridizations between strain lac18^T and these *Veillonella* species and the type species of the genus, *Veillonella parvula* DSM 2008^T, confirmed the genotypic distinctness of the novel isolate. Data from phenotypic studies also served to differentiate strain lac18^T from related strains. Therefore strain lac18^T represents a novel species of the genus *Veillonella*, for which the name *Veillonella magna* sp. nov. is proposed. The type strain is lac18^T (= CCUG 55454^T = CIP 109767^T = DSM 19857^T = JCM 15053^T).

The anaerobic, Gram-staining-negative cocci of the genus *Veillonella* Prévot (Prévot, 1933) belong phylogenetically to the *Sporomusa* sub-branch of the class *Clostridia* of the Gram-type-positive bacteria (Stackebrandt *et al.,* 1985; Willems & Collins, 1995). The taxonomy of the genus is based on extensive genotypic DNA-DNA hybridization studies performed by Mays *et al.* (1982) and reaffirmed recently by Byun *et al.* (2007). At the time of writing, the genus comprises the type species *Veillonella parvula* (Veillon & Zuber, 1898; Prévot, 1933; Mays *et al.*, 1982) and ten other species: *Veillonella alcalescens* [Lewkowicz, 1901; Prévot, 1933; subsequently reclassified as a later heterotypic synonym of *V. parvula* (Mays *et al.*, 1982)], *V. atypica*, *V. criceti*, *V. dispar*, *V. ratti*, *V. rodentium* (Rogosa, 1965; Mays *et al.*, 1982), *V. caviae* (Mays *et al.*, 1982), *V. montpellierensis* (Jumas-Bilak *et al.,* 2004), *V. denticariosi* (Byun *et al.*, 2007) and *V. rogosae* (Arif *et al.,* 2008).

The type strains of all recognized *Veillonella* species have been isolated from their main natural habitats, namely the oral cavity and the gastrointestinal tract of homeothermic vertebrates (Smith, 1965; Rogosa, 1984; Kolenbrander, 2006). The species *V. parvula*, *V. atypica*, *V. dispar*, *V. montpellierensis*, *V. denticariosi* and *V. rogosae* have been isolated from man and *V. criceti*, *V. ratti*, *V. rodentium* and *V. caviae* have been isolated from rodents. In food animals, *Veillonella* strains are also detected regularly as indigenous inhabitants of all sections of the gastrointestinal tract (Johns, 1951; Alexander & Davies, 1963; Smith & Jones, 1963; Tannock & Smith, 1970; McGillivery & Cranwell, 1992; Leser *et al.,* 2002; Tsukahara & Ushida, 2002; Murphy *et al.,* 2005; Gong *et al.,* 2007; Wise & Siragusa, 2007).

The veillonellae possess a 'truncated glycolytic system' (Rogosa *et al.,* 1965) but utilize the metabolic end products of co-existing carbohydrate-fermenting bacteria, i.e. three- and four-carbon organic acids, as energy and carbon sources via the methylmalonyl-CoA pathway (Delwiche *et al.,* 1985; Denger & Schink, 1992; Seeliger *et al.,* 2002). In the gastrointestinal tract, strains of the genus *Veillonella* thereby constitute an essential link with indigenous lactic acid bacteria in a natural microbial food-chain (Marounek & Bartos, 1987; Pacheco Delahaye *et al.*, 1994; Kolenbrander, 2006). Mixed cultures of veillonellae and, *inter alia*, lactic acid bacteria, isolated from the caeca of chickens, are inhibitory to enteropathogenic bacteria (Hinton *et al.,* 1991; Hinton *et al.,* 1992; Corrier *et al.,* 1995). One such mixed culture is commercially available as a health-promoting competitive exclusion culture for poultry production (Nisbet, 2002).

Strain lac18^T was isolated in 2007 in the course of a cultural study of lactic acid bacteria from porcine mucosal jejunum. A cross-bred fattening pig was reared at our Institute and euthanized at 62 days by means of an overdose of sodium pentobarbital, in accordance with the legal requirements of the relevant local authority for animal welfare. Fresh, washed, pea-size mucosal samples were inoculated into 10 ml of a medium selective for lactic acid bacteria and containing porcine gastric mucin (Type III; Sigma) as the main carbon and nitrogen source. This medium, henceforth referred to as mucin medium, contained the following (l^{-1}): 10.0 g mucin, 0.01 g peptone, 0.01 g yeast extract, 0.01 g glucose, 0.3 g NaCl, 0.1 g $CaCl_2$, 6.0 g KH_2PO_4, 5 ml Rogosa's salt solution (Rogosa *et al.*, 1951). 1 ml modified (lacking elements already included in Rogosa's salt solution) Pfennig's SL8 trace element solution (Bast, 2001), 0.2 ml vitamin solution (Stams *et al.*, 1993) and 0.5×10^{-3} g resazurin. All of the components were autoclaved, except the vitamins, which were filter-sterilized. The pH of the medium was adjusted to 5.0 using 37-38 % HCl prior to autoclaving. Incubations were performed in loosely screw-capped 15 ml tubes at 37 °C under anaerobic, CO_2-enriched (18 % by vol.) conditions in an anaerobic jar using Anaerocult A gas packs (Merck). The incubation time between initial transfers was always 7-14 days. Single colonies were obtained on mucin medium containing 0.4–0.75 % agar. Strain lac18^T was eventually recovered from a co-culture with an *Enterococcus* isolate (lac32) on MRS agar (Roth). It was presumptively identified to the genus level using the VITEK Anaerobe Identification system (bioMérieux). Strain lac18^T appeared to be distinct from a total of 32 isolates that were recovered from the gastric and jejunal mucosae of two pigs (data not shown). We decided, therefore, to classify and characterize strain lac18^T by means of a polyphasic approach. On the basis of the data from this study, we describe the first species of the genus *Veillonella* with a type strain of non-human or non-rodent origin.

Definitive identification of strain lac18^T, including its taxonomic assignment to the genus *Veillonella*, was obtained using 16S rRNA gene sequence analysis. Genomic DNA was extracted from cells with a NucleoSpin Tissue kit (Macherey-Nagel). The 16S rRNA gene was amplified using a HotStarTaq Master Mix kit (Qiagen) and primers 27f and 1492r (Lane, 1991). Amplified PCR products were purified using a High Pure PCR Product Purification kit (Roche) and sequenced commercially (MWG Biotech). A 16S rRNA gene sequence of 1449 bp was obtained and subsequently analysed with the Ribosomal Database Project (RDP) II classification algorithm (http://rdp.cme.msu.edu/; Maidak *et al.,* 2001; Wang *et al.,* 2007) and the BLAST algorithm (http://www.ncbi.nlm.nih.gov/; Altschul *et al.,* 1990; McGinnis & Madden, 2004). Phylogenetic analysis with MEGA software (version 4.0) (Tamura *et al.,* 2007) was conducted to infer an evolutionary tree using the neighbour-joining method (Saitou & Nei, 1987) based on the Kimura two-parameter model (Kimura, 1980). The taxonomic position of strain lac18^T in the genus *Veillonella* was further resolved by means of partial sequence (703 bp) analysis of the 70 kDa heat-shock protein gene (*dnaK*) as proposed by Marchandin *et al.* (2003). Phylogenetic analysis in BLAST was carried out as described for the 16S rRNA gene. As there is currently no published threshold for *dnaK* gene sequence-based species delineation for the genus *Veillonella* (Marchandin *et al.,* 2005), a pairwise BLAST 2 SEQUENCES analysis of all known *dnaK* sequences in the genus was also performed (http://www.ncbi.nlm.nih.gov/; Tatusova & Madden, 1999). DNA-DNA hybridization with *V. ratti* DSM 20736^T, *V. criceti* DSM 20734^T and *V. parvula* DSM 2008^T was determined using a membrane filter technique (Johnson, 1994) with digoxigenin-based products from Roche (DIG DNA labelling kit, DIG Easy Hyb granules, Anti-DIG-AP, CDP-Star) according to the manufacturer's protocols. The amount of genomic DNA immobilized on the nylon membranes was 4000 ng. The actual hybridization was performed at 42 °C for 12 h. Signal intensities were visualized by means of long exposure to a charge-coupled device camera (SensiCam Qe, CamWare software version 2.20; PCO) and the similarities (%) were determined using the PCBAS software program (version 2.09e; Raytest).

The 16S rRNA gene sequence analysis indicated that strain lac18^T was most closely related to the *V. ratti*-*V. criceti* cluster (Johnson & Harich, 1983) of the genus *Veillonella*, with *V. ratti* ATCC 17746^T (96.6 %), *V. criceti* ATCC 17747^T (96.6 %), *V. denticariosi* RBV106^T (95.1 %) and *V. atypica* ATCC 17744^T (95.1 %) as the closest relatives with validly published names. The sequence similarity with respect to *V. parvula* ATCC 10790^T was 94.0 %. High similarity (99.4 %) was obtained with the 1309 bp sequence of one unpublished isolate, *Veillonella* sp. MY-P9 (GenBank accession no. DQ979378 deposited by M. Y. Jung & Y.-H. Chang, 2006). The genetic distinctness of strain lac18^T with respect to the *V. ratti*-*V. criceti* cluster was strongly supported by a high bootstrap value (100 %) in the neighbour-joining analysis (Fig. 1). The *dnaK* gene sequence analysis indicated that the taxonomic position of

the novel strain was closer to *V. ratti* ATCC 17746^T (84.5 %) than to *V. criceti* ATCC 17747^T (83.4 %). The sequence similarity of the novel strain was highest with the *Veillonella* sp. isolate MY-P9 (99.7 %). The BLAST 2 SEQUENCES analysis showed that the interspecific similarity of the *dnaK* gene sequences of recognized strains in the genus *Veillonella* (except *Veillonella* sp. ADV 4313.2, *Veillonella* sp. ADV 360.1 and *Veillonella* sp. MY-P9) varied between 77.9 % (lac18^T versus *V. dispar* ATCC 17748^T) and 95.7 % (*V. denticariosi* RBV81 versus *V. rodentium* ATCC 17743^T). The intraspecific similarities of the *dnaK* gene sequences of strains of recognized species varied between 98.4 % and 99.8 % (*V. montpellierensis*). This comparison produced a 96–98 % threshold for *dnaK* gene-based species delineation in the genus *Veillonella* (see Supplementary Table S1, available in IJSEM Online). DNA-DNA-hybridization results confirmed the genotypic distinctness of strain lac18^T within the genus *Veillonella*: the DNA-DNA relatedness values with *V. ratti* DSM 20736^T, *V. criceti* DSM 20734^T and *V. parvula* DSM 2008^T were 40.0, 39.0 and 27.0 %, respectively.

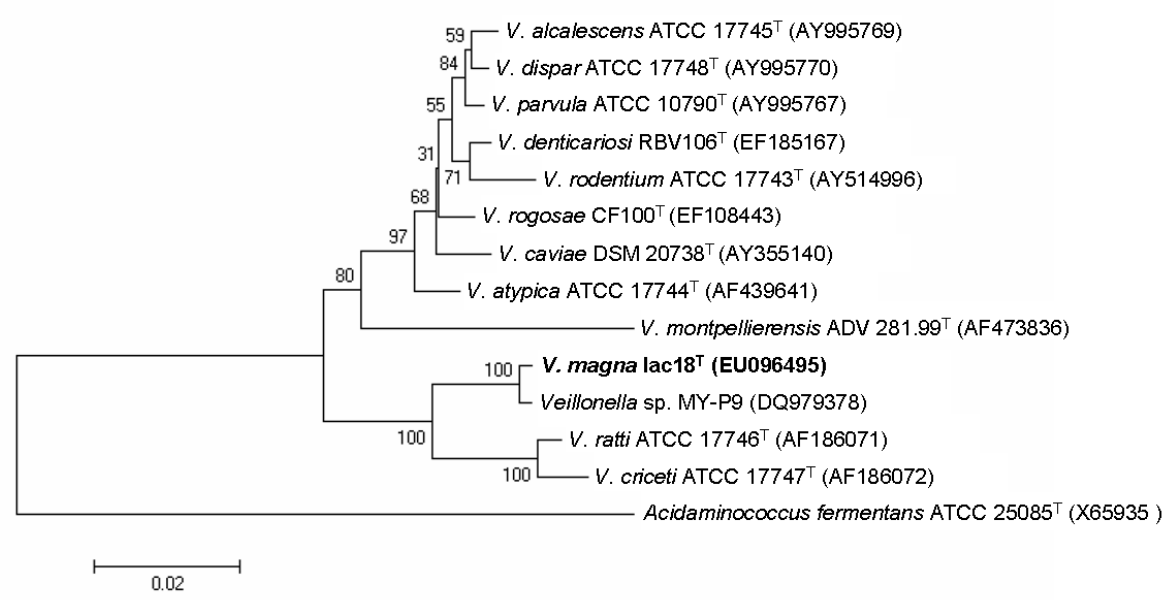

Fig. 1. Neighbour-joining phylogenetic tree, based on partial 16S rRNA gene sequences, showing the relationships between strain lac18^T (1449 bp), all type strains of the genus *Veillonella* and isolate *Veillonella* sp. MY-P9. *Acidaminococcus fermentans* ATCC 25085^T (1488 bp) was used as the outgroup organism. Bootstrap percentages (based on 1000 replicates) are shown at nodes. Bar, genetic distance of 2 %.

Supplementary Table S1. Intra- and interspecific (%) similarity values of the *dnaK* gene sequences of strain *Veillonella magna* lac18^T (703 bp) and other strains in the genus *Veillonella* as obtained by pairwise BLAST 2 SEQUENCES analysis

The *dnaK* sequences from strains other than *Veillonella magna* lac18^T were retrieved from GenBank/EMBL/DDBJ with the following accession numbers: AY514999, *V. parvula* ATCC 17745; AF440437, *V. parvula* CIP 60.1; AY220521, *Veillonella* sp. ADV 360.1; AF440435, *V. dispar* ATCC 17748^T; AF440436, *V. atypica* ATCC 17744^T; AY353714, *V. caviae* ATCC 33540^T; AY514997, *V. rodentium* ATCC 17743^T; EF219278, *V. denticariosi* RBV106^T; EF219279, *V. denticariosi* RBV81; AY353717, *V. montpellierensis* ADV 281.99^T; AY353715, *V. montpellierensis* ADV 2216.03; AY353716, *V. montpellierensis* ADV 3198.03; AY353713, *V. criceti* ATCC 17747^T; AY514998 (590 bp), *V. ratti* ATCC 17746^T; AY353711 (621 bp), *V. ratti* DSM 20736^T; AY793381, *Veillonella* sp. ADV 4313.2; EF392691, *Veillonella* sp. MY-P9. The GenBank/EMBL/DDBJ accession number of the *dnaK* gene sequence of strain *Veillonella magna* lac18^T is EU096494. Values used for definition of a threshold range for species delineation are in dark grey (intraspecific) and light grey (interspecific), respectively.

Strain		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*V. parvula* ATCC 17745	99.3	97.8	95.2	90.8	88.3	86.2	85.4	85.8	82.5	82.5	84.4	81.2	80.1	79.9	81.7	79.3	79.3
2	*V. parvula* CIP 60.1		98.2	95.2	93.9	88.0	85.7	84.8	85.2	85.0	84.6	84.4	80.5	81.2	80.5	81.7	78.3	78.9
3	*Veillonella* sp. ADV 360.1			95.9	94.3	88.7	87.4	85.7	86.1	86.2	85.8	85.5	82.5	81.1	80.5	81.6	78.5	79.1
4	*V. dispar* ATCC 17748^T				94.3	88.5	87.1	85.6	86.0	86.8	86.8	85.8	82.7	81.0	80.3	80.7	78.9	77.9
5	*V. atypica* ATCC 17744^T					86.1	86.3	84.4	84.4	85.0	84.6	85.5	83.9	80.6	79.9	81.8	79.3	79.8
6	*V. caviae* ATCC 33540^T						86.3	85.7	86.1	84.2	84.3	86.5	82.3	81.2	80.9	81.5	78.4	78.6
7	*V. rodentium* ATCC 17743^T							95.2	95.7	82.1	82.1	81.8	81.8	79.9	79.3	80.0	78.6	78.4
8	*V. denticariosi* RBV106^T								99.5	80.8	80.8	81.5	81.3	81.1	80.2	80.2	78.5	78.5
9	*V. denticariosi* RBV81									80.8	80.8	81.5	81.4	81.4	80.5	80.6	78.6	78.6
10	*V. montpellierensis* ADV 281.99^T										99.8	98.4	82.5	79.4	79.0	80.2	78.4	78.5
11	*V. montpellierensis* ADV 2216.03											98.4	82.9	79.4	79.0	80.4	78.2	78.7
12	*V. montpellierensis* ADV 3198.03												83.0	80.3	79.5	80.9	79.5	79.5
13	*V. criceti* ATCC 17747^T													85.3	84.6	87.3	83.3	83.4
14	*V. ratti* ATCC 17746^T (AY514998)														99.5	90.9	85.2	85.0
15	*V. ratti* DSM 20736^T (AY353711)															90.2	84.5	84.5
16	*Veillonella* sp. ADV 4313.2																86.2	86.4
17	*Veillonella* sp. MY-P9																	99.7
18	*V. magna* lac18^T																	-

Phenotypic studies on strain lac18^T were conducted mainly in comparison with *V. ratti* DSM 20736^T and *V. criceti* DSM 20734^T (which were purchased, for this purpose, from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Unless stated otherwise, the standard cultivation medium used was a modified version of *Veillonella* medium (Rogosa, 1956) (i.e. without antibiotic and basic fuchsin and supplemented with 0.001 g resazurin l^{-1} and 0.003 g putrescine l^{-1}).

Cell morphology was observed under a Zeiss light microscope at × 1000 magnification and under a digital scanning electron microscope (DSM 950; Zeiss) at × 5000-20 000 (Taras, 2001). Gram-staining was performed using a Gram-colour staining set (Merck) according to the manufacturer's instructions. Spore formation and motility were analysed using conventional methods (Bast, 2001). Commercial tests (Fluka) were applied for the detection of cytochrome oxidase and nitrate reduction. The benzidine reaction with porphyrin and catalase activity were assayed according to Deibel & Evans (1960). The production of H_2O_2 was studied as described by Juárez Tomás *et al.* (2004) with the following exceptions: prolonged incubation was performed under both anaerobic and microaerobic (8-10 % CO_2, 5-7 % O_2, by vol.; Anaerocult C gas packs, Merck) conditions for up to 4 days and exposure to air was for 24 h. The H_2O_2 assays were evaluated as described by Otero & Nader-Macías (2006). H_2S production was analysed in a defined modified *Veillonella* medium with 0.6×10^{-3} M L-cysteine (Rogosa & Bishop, 1964). For biochemical profiling, the Rapid ID 32A and API ZYM systems (bioMérieux) were used according to the manufacturer's instructions. Blood agar base No. 2 (LabM) plus 5 % defibrinated horse blood was chosen as the second culture medium with Rapid ID 32A. Putrescine auxotrophy was analysed in the defined modified *Veillonella* medium, supplemented with putrescine at 0.003 and 0.005 g l^{-1} (Rogosa & Bishop, 1964a; Ritchey & Delwiche, 1975). Fermentation of fructose was studied likewise using supplementation with 0.02 M D-fructose. Short-chain fatty acids produced as metabolic end products from the fermentation of lactate were determined as described previously (Schäfer, 1995) and gas production was assayed by measuring increases in pressure in air-tight, screw-capped roll tubes. Growth characteristics under various pH and temperature values were studied using standard anaerobic methods (Hungate, 1969). The response to oxygen was assessed using aerobic and microaerobic (Anaerocult C gas packs) incubation with or without the reducing agent sodium thioglycolate (0.75 g l^{-1}).

The results of the phenotypic characterization, including differential features, are given in the species description. Phenotypic characteristics that serve to differentiate strain lac18^T from *V. ratti* DSM 20736^T and *V. criceti* DSM 20734^T are given in Table 1. On the basis of the results of 16S rRNA and *dnaK* gene sequencing and the differential phenotypic data, strain lac18^T

represents a novel species of the genus *Veillonella*, for which the name *Veillonella magna* is proposed.

Table 1. Results of differential phenotypic characterization of strain lac18^T, *V. ratti* DSM 20736^T and *V. criceti* DSM 20734^T

Taxa: 1, Strain lac18^T; 2, *V. ratti* DSM 20736^T; 3, *V. criceti* DSM 20734^T. +, Positive; -, negative; w, weak. In the API ZYM, all strains were negative for the following: lipase (C 14), cystine arylamidase, trypsin, *α*-chymotrypsin, *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *α*-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase, α-fucosidase. Values for the API ZYM are means of the activity mark±SD, *n*=10. The activity mark is a measure of the quantity of hydrolysed substrate (nmol): 0=<5; 1=5-<10; 2=10-<20; 3=20-<30; 4=30-<40; 5=≥40.

Characteristic	**1**	**2**	**3**
Cell diameter (μm)	0.65-0.85	0.3–0.5*	0.3–0.5*
Catalase	- or delayed and w	+	+
Accumulation of H_2O_2			
Anaerobic	w	-	-
Microaerobic	Moderate	w	w
Biochemical profile (API ZYM)			
Alkaline phosphatase	3.4 ± 0.7	2.3 ± 1.0	2.5 ± 0.5
Esterase (C4)	1.1 ± 0.5	1.4 ± 0.5	2.2 ± 0.4
Esterase lipase (C 8)	0.1 ± 0.3	0.9 ± 0.3	1.1 ± 0.3
Leucine arylamidase	1.4 ± 0.8	1.3 ± 0.9	0.6 ± 0.5
Valine arylamidase	0.1 ± 0.3	0.2 ± 0.4	0.3 ± 0.5
Acid phosphatase	4.1 ± 0.5	4.1 ± 0.9	3.8 ± 0.4
Naphtol-AS-Bl-phosphohydrolase	3.3 ± 0.5	4.7 ± 0.5	3.4 ± 0.7
Putrescine requirement	+	-*	+
Fermentation of fructose			
Change of pH at 28 days†	-0.01±0.01	-0.29±0.09	-0.32±0.02
Temperature range for growth (on modified *Veillonella* medium)			
45 °C (agar)	+	-	-
40 °C (broth), OD_{max}†‡	1.38±0.04	0.87±0.02	0.88±0.02

*Data taken from Holt *et al.* (1994); cell diameter as generally given for the genus *Veillonella*.

†Values are means±SD, *n*=3.

‡OD was measured at 600 nm with an Ultrospec 2000 spectrophotometer (Pharmacia Biotech).

Description of *Veillonella magna* sp. nov.

Veillonella magna (mag'na. L. fem. adj. *magna* big, referring to the cell size in comparison with other species of the genus *Veillonella*).

Cells are spherical to coccoid, 0.65-0.85 μm in diameter (mean, approx. 0.75 μm) and are arranged singly or in pairs (rarely in short chains or small masses). Adjacent sides of cell pairs are flattened and the cell surface is convoluted (Fig. 2). Cells stain Gram-negative, with an enhanced tendency to resist decolorization after 48 h incubation. They are non-spore-forming and non-motile. After anaerobic incubation on modified *Veillonella* agar at 37 °C for 24 h, colonies are circular, up to 1.2 mm in diameter, have entire margins and smooth, shiny surfaces and are raised, opaque, creamy grey, soft and moist. After 48 h, colonies look similar but are up to 2.0 mm in diameter and greyish beige. Cells are non-haemolytic on blood agar. Cytochrome oxidase is not present. Nitrate is reduced. The benzidine test for porphyrin is positive. Catalase activity is negative or delayed (approx. 30-45 s) and weak. The type of catalase is not determined in the benzidine test. Benzidine reacts non-specifically with the porphyrin compounds of the haem group in respiratory systems including (besides catalase) cytochromes and nitrite reductase (Bascomb & Manafi, 1998). H_2O_2 accumulation is weak under anaerobic conditions and moderate under microaerobic conditions. H_2S is produced from L-cysteine. Biochemical profiling with Rapid ID 32A yields positive results for arginine dihydrolase, reduction of nitrates, alkaline phosphatase and histidine arylamidase (identification profile 2000500001). The leucine arylamidase reaction is ambiguous using modified *Veillonella* medium [identification profile 200050(0/2)001]. API ZYM results are indicated in Table 1. Putrescine is required for normal growth. Negative for fermentation of fructose. Lactate is metabolized anaerobically to propionate and less acetate. Gas is also produced, often abundantly. The temperature range for growth is approximately 21–45 °C. Growth is very weak at approximately 21 °C, absent at 46 °C, good at 30 °C and very good at 37 °C and 40 °C. The pH range for growth is 5.5–9.5 (optimum, pH 6.5–7.5). Alkalinization of modified *Veillonella* medium occurs at pH 5.5–6.0 (maximally to pH 6.5) and acidification occurs at pH 6.5-9.5 (maximally to pH 6.2). Cells are anaerobic and microaerotolerant (less than approx. 5 % O_2, by vol.), showing no growth on agar plates but producing vigorous growth and long-lasting viability (up to at least 2 weeks) in broth medium suspended in air-tight, screw-capped roll tubes under aerobic, unreduced conditions at 37 °C.

The type strain, lac18^T (= CCUG 55454^T = CIP 109767^T = DSM 19857^T = JCM 15053^T), was isolated from the mucosal jejunum of a healthy pig in Berlin, Germany.

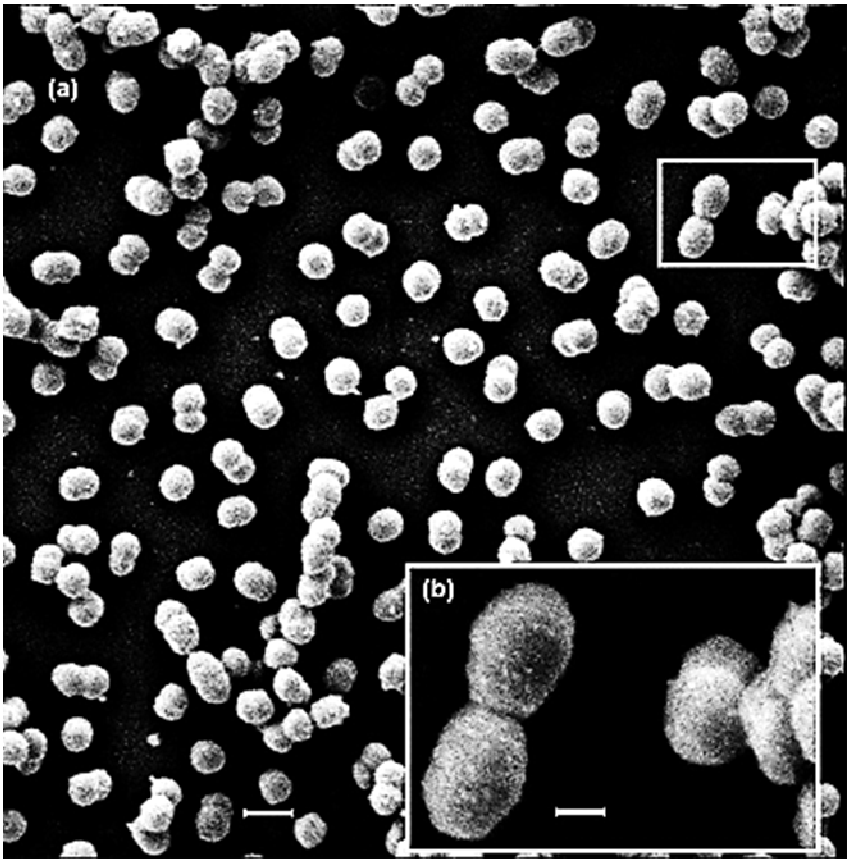

Fig. 2. **Scanning electron micrographs of strain lac18^T grown on modified *Veillonella* agar for 22 h under anaerobic conditions at 37 °C. Bars, 1000 nm (a) and 250 nm (b).**

Emended description of *Veillonella ratti* (Rogosa 1965) Mays *et al.* 1982

The description remains as given by Rogosa (1984) except that the strain *V. ratti* DSM 20736^T is capable of fructose fermentation.

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Chapter VII

Lactic acid-related bacterial community from the mucosal stomach and jejunum of a pig, including *Olsenella umbonata* sp. nov. and *Veillonella magna* sp. nov.

Kraatz, M. (2010). Lactic acid-related bacterial community from the mucosal stomach and jejunum of a pig, including *Olsenella umbonata* sp. nov. and *Veillonella magna* sp. nov. Poster presentation at the 7th Joint Symposium Rowett-INRA 2010 'Gut Microbiology: new Insights into Gut Microbial Ecosystems', Aberdeen, Scotland (UK), June 23-25, 2010.

Abstract

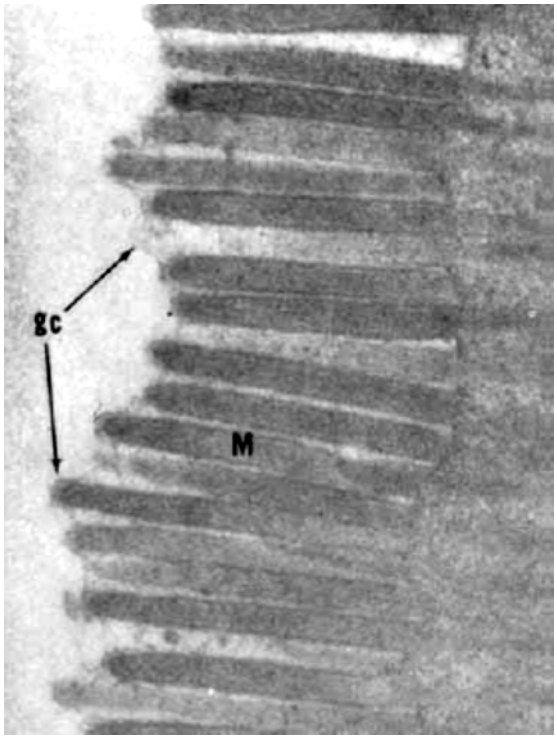
In the proximal gastrointestinal (GI) tract of pigs, the resident, indigenous microbiota are normally restricted to the mucosal microhabitats. Mucosa-associated bacteria are selected by their co-evolved ability to establish microcolonies in the microaerobic milieu of the epithelial cell surface and/or the covering mucus layer. Mucin glycoproteins are the principal constituents of the jejunal brush-border glycocalyx and mucus. Providing multiple niches for diverse and cooperative bacterial communities, they accomplish an ecologically outstanding stabiliser function in the interdigestive proximal GI tract. I applied a newly created porcine gastric mucin (PGM) based medium in the specific isolation of lactic acid-related bacteria (LARB) from the porcine mucosal stomach and jejunum. 14 or 13 isolates were obtained using microaerobic or anaerobic conditions, respectively, and partially identified by means of a 16S rRNA gene sequence-based polyphasic approach. The isolated LARB community was complex and phylogenetically diverse: It comprised all three groups of the *Firmicutes* and *Actinobacteria*, i.e. the functionally redundant lactic acid-producing bacteria *sensu stricto* and *sensu lato* (lactobacilli, enterococci and *Olsenella umbonata* [1]) and the metabolically linked lactic acid-fermenting bacteria (*Veillonella magna* [2]). The two novel species are presumably indigenous to the mucosal GI microhabitats. They are microaerotolerant (moderately obligate) anaerobic and capable of utilising PGM. Proteolytic activity in the concerted fermentation of mucins (*O. umbonata*) and disposal of accruing lactic acid (*V. magna*) present their likely ecological niches *in vivo*.

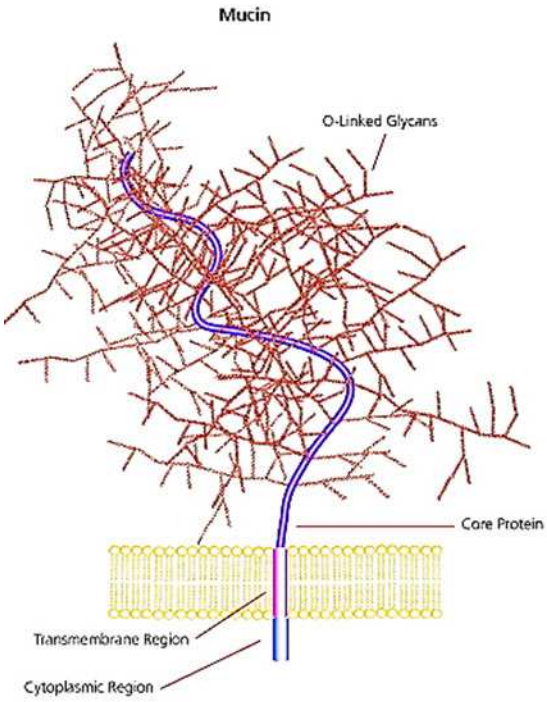
[1] Kraatz M; Wallace R J; Svensson L. (submitted). Int J Syst Evol Microbiol.

[2] Kraatz M; Taras D. (2008). Int J Syst Evol Microbiol 58, 2755-2761.

Introduction

In the pig stomach and small intestine, the mutualistic symbiosis with the indigenous gut microbiota tends towards antagonism (Richards *et al.,* 2005). Host defence factors select for lactobacilli and other lactic acid-producing bacteria (LAB) that are capable of colonising the microaerobic mucosa-associated microhabitats. LAB form food webs with lactic acid-fermenting bacteria in microcolony and biofilm communities of lactic acid-related bacteria (LARB) at the epithelial cell surfaces and in the covering mucus gel layers (Tannock, 2005; Wilson, 2005; Walter, 2008).





Left: Surface of an absorptive epithelial cell from the jejunum of a 3-wk-old pig. M, microvilli; gc, covering of glycocalyx (Staley *et al.,* 1968).

Right: Complex structure of mucin (Sigma-Aldrich, 2010).

During interdigestive periods, in the absence of readily assimilable substrates, the trophic chain of LARB is sustained through lactic fermentation of host mucin glycoproteins (Bradshaw *et al.,* 1994). Mucins are the principal macromolecular constituents of the jejunal brush-border glycocalyx and mucus (Wilson, 2005). Providing multiple metabolic sites (niches) for diverse and complexly cooperative bacterial communities, they accomplish an ecologically outstanding stabiliser function in the proximal gastrointestinal (GI) tract (Hoskins, 1992).

Objectives

I aimed at answering the prior question in gut microbial ecology: 'Whose habitat is this?' (Tannock, 2008).

I applied a newly created habitat-simulating cultural approach on samples from the mucosal stomach and jejunum of a 9-wk-old healthy domestic pig.

My objectives were as follows:

1. Specific isolation of a complex community of LARB
2. Isolation of strains of novel species
3. Polyphasic taxonomic analysis and description of novel species.

Materials and Methods

Specific isolation of LARB

I inoculated fresh, washed mucosal samples in 10 ml of PGM-plus medium. The main composition of the medium was as follows (l^{-1}): 10.0 g PGM (porcine gastric mucin; Sigma), 0.01 g each of peptone, yeast extract and glucose (PYG), 0.575 g $MgSO_4$ and 0.14 g $MnSO_4$ (Rogosa *et al.,* 1951) as elective and 37-38 % HCl (pH 5.0) as selective agents. I used the following conditions: 37 °C, an- or microoxic CO_2 atmosphere, 7-14 d (enrichment). Bacteria were sub-cultured on solid PGM-plus and soft PGM (PGM-plus without PYG). I obtained pure cultures on conventional complex media.

Polyphasic identification of isolates

I assigned the isolates to groups following a basic phenotypic analysis including VITEK ANI (bioMérieux). Finally, I performed a 16S rRNA gene sequence-based phylogenetic analysis of group-representative isolates.

Polyphasic taxonomic analysis of strains of novel species

Basic phenotypic and genotypic analysis: This included a *dnaK* gene sequence-based phylogenetic analysis, DNA-DNA hybridizations, scanning electron microscopy, biochemical and enzyme profiling using conventional methods and API kits (bioMérieux) and the determination of metabolic end products and cellular fatty acids.

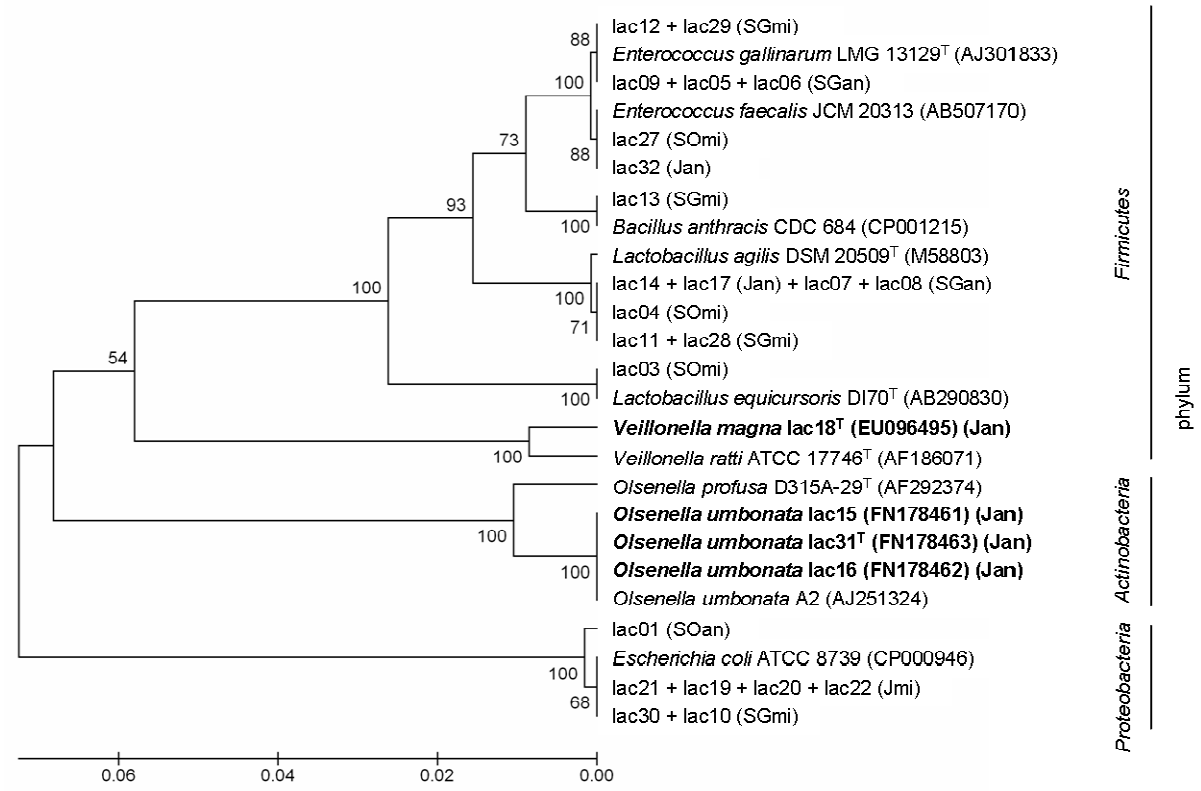
Determination of ecological status: I carried out a phenotypic analysis of the temperature and pH range for growth, O_2 relationship, accumulation of H_2O_2, bile resistance and growth on PGM.

Results

Polyphasic identification of isolates

I obtained a total of 27 bacterial isolates of seven phenotypic groups.

The isolated community was principally adapted to the microoxic conditions of the mucosa-associated GI microhabitats. Members of all groups were mesophilic and facultatively to microaerotolerant anaerobic. Most of the isolates exhibited mucinolytic glycosidases.



UPGMA tree of group-representative partial 16S rRNA gene sequences showing the phylogenetic relationship of mucosal isolates (strains of novel species in bold) and most closely related recognised strains. Accession numbers and origins of the newly isolated strains are given in parentheses (SO, SG, J, isolated from oesophageal stomach, glandular stomach, jejunum, respectively; an, mi, isolated under anoxic, microoxic conditions, respectively). Numbers at branch points are bootstrap values (%) based on 1000 replicates. Scale bar, 0-6 % sequence divergence.

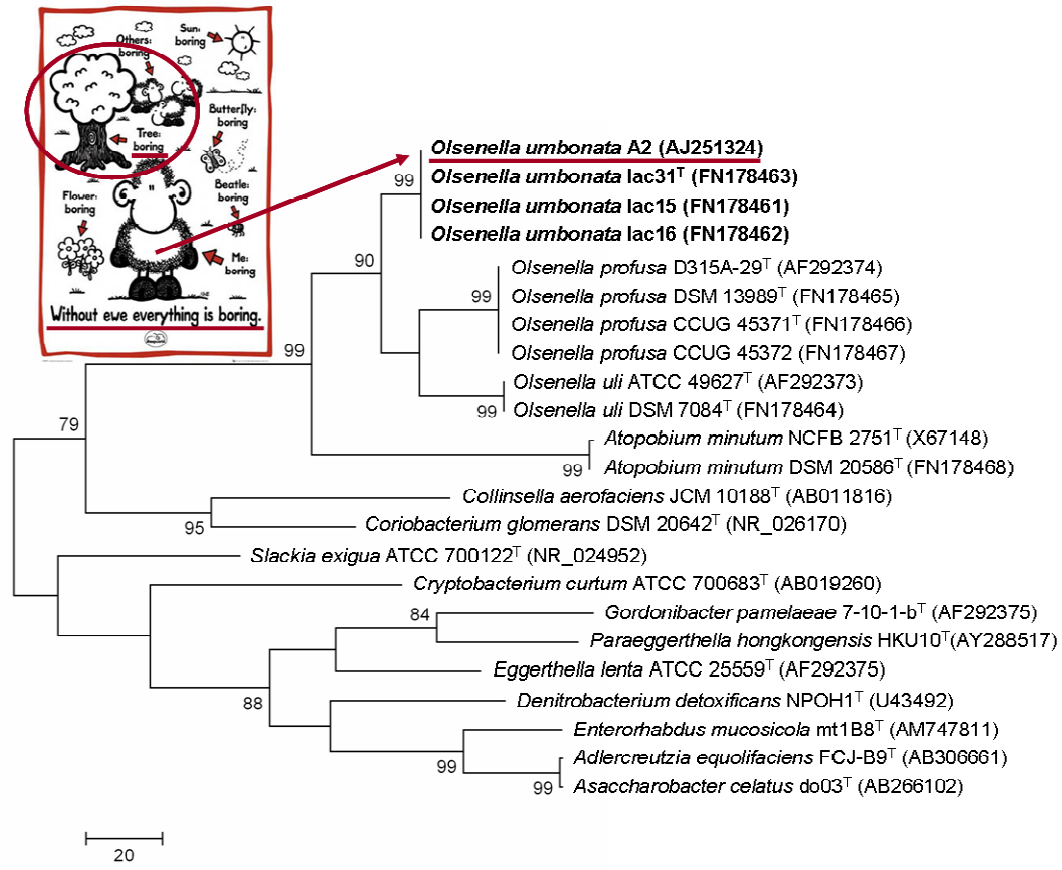
The isolated community was complex and phylogenetically diverse: It comprised all three groups of LARB of the phyla *Firmicutes* and *Actinobacteria*, namely the functionally redundant LAB *sensu stricto* (genera *Enterococcus*, *Lactobacillus*) and *sensu lato* (genera *Bacillus* and *Olsenella*) and the lactic acid-fermenting bacteria (genus *Veillonella*).

Polyphasic taxonomic analysis of strains of novel species

The phylogenetic analysis indicated that five of the total 20 LARB isolates represented three novel species.

Strain lac03 presumably belongs to *Lactobacillus equicursoris* Morita *et al.* 2010. I have proposed the names *Olsenella umbonata* sp. nov. and *Veillonella magna* sp. nov. to accommodate the strains lac15, lac16, lac31^T and lac18^T, respectively (Kraatz & Taras, 2008; Kraatz *et al.,* in press).

My *Olsenella* strains formed a genetically coherent group with the sheep rumen isolate A2 from the Rowett Institute of Nutrition and Health. Formerly known as '*Olsenella* (basonym *Atopobium*) *oviles*', A2 is now included in the species *Olsenella umbonata* (Kraatz *et al.,* in press).



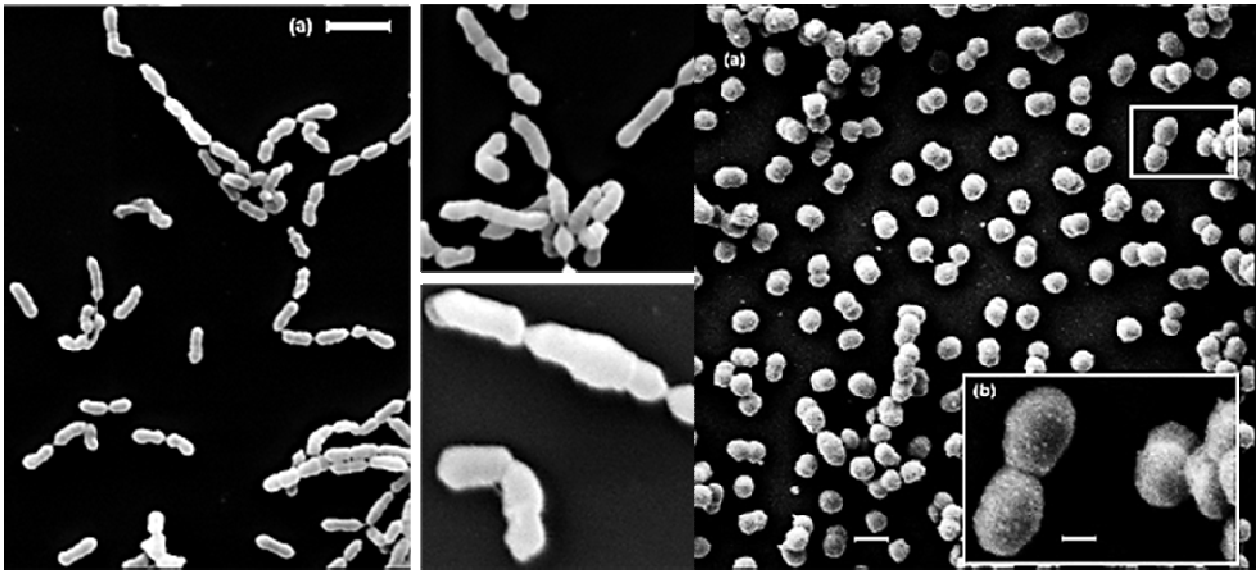
Maximum-parsimony tree of partial 16S rRNA gene sequences showing the phylogenetic relationship of the strains lac15, lac16, lac31^T, A2 (in bold) to other members of the *Olsenella-Atopobium* bigeneric branch and the actinobacterial family *Coriobacteriaceae*, including all type species. The tree is the unrooted consensus of 1000 bootstrap replicates. Bootstrap values (%) greater than 70 are given at branch points. Bar, 20 base changes between nodes. Accession numbers are given in parentheses (Sheepworld, 2010; Kraatz *et al.,* in press).

A2 and the four novel *Olsenella* and *Veillonella* strains were mesophilic, neutrophilic, microaerotolerant (moderately obligate) anaerobic and mucin-utilising. Characteristically, the olsenellae were acidotolerant, bile resistant and markedly peptidolytic LAB. *V. magna* lac18^T specifically metabolised lactic acid to propionic and acetic acids (Kraatz & Taras 2008; Kraatz *et al.,* in press).

Characteristic	*Olsenella umbonata*				*Veillonella magna*
	lac15	lac16	lac31^T	A2	lac18^T
Temperature range for growth (°C)	> 21 - ≥ 45				21 - 45
pH range for growth	4.5 - 8.0			4.5 - 8.5	5.5 - 9.5
pH optimum	6.0 - 7.0				6.5 - 7.5
Microaerotolerance (< approx. 5 % O_2)	+				+
Accumulation of H_2O_2 (an- / microaerobic)	- / ND				weak / moderate
Growth in 20 % bile / on PGM	+ / +				ND / +
α- / β-Glucosidase	+ / -				- / -
Acidification of carbohydrates (n positive / n tested)	6 / 18			5 / 18	0 / 3
	glucose, fructose, maltose, mannose, sucrose				
	trehalose				
Protease (Gelatin hydrolysis)	+				ND
Exopeptidases (n positive / n tested)	12 / 14				3 / 14
Metabolic end products (an- / microaerobic; mmol l^{-1})					
D-Lactic acid	42 / 42			35 / 35	ND / ND
Propionic acid	0 / 0				61 / 45
Acetic acid	3 / 2				48 / 31
Formic acid	4 / 2			5 / 3	ND / ND
Ammonium	14 / 12			10 / 6	+ / ND
Gas	- / ND				+ / ND

Ecologically relevant results of the phenotypic characterisation of strains of novel LARB species. ND, not determined.

Outgrown colonies of the strains of *O. umbonata* displayed distinct umbonate elevations on solid culture media. *V. magna* lac18^T was decisively different from other veillonellae due to its greater cell diameter (0.6-0.8 versus 0.3-0.5 µm) (Kraatz & Taras, 2008; Kraatz *et al.,* in press).



Bar: (a) 2 µm; (b) 1 µm; (c) 0.5 µm. **Bar: (a) 1 µm; (b) 0.25 µm.**

Scanning electron micrographs of cells of strains lac31^T (left) and lac18^T (right) grown anaerobically on PYG (48 h) and modified *Veillonella* agar (22 h), respectively (Kraatz & Taras, 2008; Kraatz *et al.,* in press).

Conclusions

PGM is a suitable basal component in the enrichment isolation of complex communities of LARB, including strains of novel species, from the mucosae of the pig proximal GI tract.

Presumably due to its ecological stabiliser function, PGM prevents overgrowth of more fastidious actinobacterial LAB *sensu lato* and metabolically subordinate lactic acid-fermenting bacteria.

O. umbonata and *V. magna* are well adapted to the mucosal microhabitats of the pig GI tract.

As other olsenellae and veillonellae, these novel species are presumably indigenous to the guts of pigs and other homeothermic vertebrates.

Proteolytic activity in the concerted degradation and fermentation of mucins (*O. umbonata*) and disposal of accruing lactic acid (*V. magna*) present their likely ecological niches in the pig proximal GI tract.

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Chapter VIII

General discussion

1. Detection of strains of novel species

1.1 Composition of the pig proximal gut microbiota

As introduced in Chapters I and II, 400 to 10000 different bacterial species can be estimated to be present in the pig gastrointestinal (GI) tract (Leser *et al.,* 2002; Walter, 2008). Despite the 'special foregut association' (Walter, 2008), the species richness is generally lower in proximal habitats due to restrictive host defence factors (Tannock, 1995; Wilson, 2005). Hence only 86 of in total 375 species-level phylotypes were detected in luminal contents from the pig ileum by Leser *et al.* (2002). The members of the mammalian faecal and pig intestinal microbiota have been assigned to 17 and 13 bacterial phyla (major phylogenetic lineages), respectively (Leser *et al.*, 2002; Ley *et al.,* 2008). However just seven phyla, namely the *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes*, *Fusobacteria* and *Chlamydiae*, comprise the majority of phylotypes in the pig gut (Wieler, 2007), with the *Firmicutes* and, in the large intestine and faeces, also *Bacteroidetes* being numerically dominant (Smith, 1965; Leser *et al.*, 2002; Richards *et al.,* 2005; Ley *et al.,* 2008; Thompson *et al.,* 2008; Leser & Mølbak, 2009).

In young pigs, the last phase of the development of a complex and relatively stable microbiota proceeds to about two to three weeks after completion of weaning and is apparently more rapid in the proximal than in the distal GI tract (Bateup *et al.,* 1998; Bomba *et al.,* 2006; Goméz, 2006). The pig from the study of Chapter IV was nine weeks old and approximately one month post weaning. Therefore it can be assumed that the bacterial communities of its stomach and jejunum exhibited adult patterns at the time point of sampling.

The phylogenetic composition of the bacterial community of Chapter IV conformed to general expectations. It was numerically dominated by isolates of the *Firmicutes* (*n*=17), with *Proteobacteria* (*n*=7) constituting the second most abundant group and *Bacteroidetes* being absent. The recovery of three isolates of the *Actinobacteria* (strains of *Olsenella umbonata*) matched the assumption that the phylogenetic range is greater in the proximal than in the distal GI tract (Turroni *et al.,* 2008).

1.1.1 Olsenellae in the pig and sheep proximal gut

Olsenellae and other bacteria of the phylum *Actinobacteria*, class *Actinobacteria* constitute a minor part of the gut microbiota (Tannock, 2008). In the study of Leser *et al.* (2002), four infrequent phylotypes of in total 375 belonged to the phylogenetic lineage of high-G+C-content bacteria, whereby one phylotype presumably represented the genus *Olsenella*. Using analysis by FCM-FISH on samples from the pig stomach and small intestine, Collado & Sanz (2007) found that members of the *Atopobium* group constituted a significant proportion of the mucosa-associated microbiota. Since the occurrence of olsenellae in the pig GI tract had been indicated by the cited and two other (Tsukahara & Ushida, 2002; Dowd *et al.*, 2008) molecular genetic studies and also by a culture-based study of pig manure (Whitehead *et al.,* 2008), the isolation of strains of *O. umbonata* was not completely surprising. It can be assumed that olsenellae, as other actinobacteria in the gut, play a relevant ecological role that is underestimated due to their relatively low numbers and often metabolically quiescent state (Tannock, 2008; Turroni *et al.,* 2008; Fakhry *et al.,* 2009).

It has been assumed that the overall gut microbiota compositions of pigs and ruminants are very different (Bomba *et al.,* 2006). In contrast to this, the results of Chapter V showed that *O. umbonata* is not strictly host species-specific but presumably indigenous to the GI tract of both pigs and sheep. Lactic acid-producing bacteria (LAB) constitute a minor fraction in adult pasture-fed ruminants and occur more often in young animals and in animals fed concentrate-supplemented diets (Stewart, 1992; Hammes & Hertel, 2009). As summarised in Chapter V, the rumen of concentrate-fed cattle had for some time been recognised as a habitat of olsenellae. Eschenlauer *et al.* (2002) isolated strain A2 of *O. umbonata* together with two phenotypically identical strains from highly (10^6) diluted ruminal fluids of two different concentrate-fed sheep. This indicates that *O. umbonata* is a common and relatively abundant member of the sheep gut microbiota in intensive production systems.

As implicated by considerable differences in the cellular fatty acid compositions of strain A2 and the strains from pig jejunum (Chapter V), the species *O. umbonata* may be in a process of host-driven adaptive diversification into distinct subpopulations, comparable to what has recently been observed for *Lactobacillus reuteri* (Oh *et al.,* 2010). The four novel strains of *O. umbonata* exhibited 16S rRNA gene-sequence similarities of 99.1 % and 99.8 % to uncultured bacterium clones from municipal wastewater sludge and human faeces, respectively (Chapter V, unpublished data). Therefore the habitats of this species seem to include humans and are likely more diverse than is presently known.

1.1.2 Veillonellae in the pig proximal gut

Promoted by their syntrophic dependence on co-existing LAB, asaccharolytic lactic acid-fermenting veillonellae and megasphaerae of the phylum *Firmicutes*, class *Negativicutes* (former *Sporomusa* sub-branch of the class *Clostridia*) (Marchandin *et al.,* 2010) have long been recognised as regular inhabitants of the pig GI tract (Kenworthy, 1973; reviewed in Chapters II and VI). Whereas *Megasphaera elsdenii* is assumed to be mainly responsible for lactic acid utilisation in the large intestine, members of the genus *Veillonella* seem to be indigenous to a wider range of habitats, including the pig mucosal proximal gut (Duncan *et al.,* 2004; reviewed in Chapter VI). However, despite their wider distribution, veillonellae constitute only a numerically minor part of the pig gut microbiota (Smith, 1965; Leser *et al.*, 2002). Against this background, it can be concluded that the isolation of *Veillonella magna* lac18^T was fostered by the election of LAB and particularly *Enterococcus* strain lac32. Enterococci and other LAB are a potential source not only of growth-enhancing lactic acid, but also of the diamine putrescine (Wilson, 2005; Goldin, 2006; Lebeer *et al.,* 2008), for which, according to the results of Chapter VI, *V. magna* lac18^T is auxotroph.

V. magna is the first *Veillonella* species that was originally isolated from the pig GI tract. From the results of both 16S rRNA and *dnaK* gene sequence-based phylogenetic analyses, *V. magna* lac18^T was very closely related only to the strain *Veillonella* sp. MY-P9 from the pig intestine (Chang, 2008; Chapter VI). This indicates that *V. magna*, in contrast to some other veillonellae, is an indigenous member of the pig gut microbiota and exhibits some degree of host species specificity.

2. Ecological status: indigeneity

2.1 Individuality of the proximal gut microbiota

The conclusion that *O. umbonata* and *V. magna* are indigenous to the pig GI tract (Chapter VII) implies, by definition, that the novel species are normally present and resident in all pigs due to an adaptive co-evolution. However, it is well established that, at the finer scales of species and strains, their exists a high individuality of the composition of the predominant indigenous communities among members of a host species (Tannock, 2005; Tannock, 2008; Thompson *et al.*, 2008; Turroni *et al.*, 2008; Camp *et al.,* 2009). This uniqueness to individuals is primarily due to a host genotype impact that is conferred by specific mucin chemotype patterns or, more precisely, by the blood-group antigenic determinants of the mucin oligosaccharide side chains (Gaskins *et al.,* 2008). The ecological primacy of host mucin genetic determinants gives an explanation why the individuality of the indigenous

microbiota of monogastric mammals is more distinct in the mucosa-associated habitats of the proximal gut than in the luminal habitats of the distal gut (Reuter, 2001; Leser & Mølbak, 2009). The host-specific mucin chemotypes may influence the ecological fitness of different strains of species via selection for 'niche-specific genes' (Walter, 2005) for bacterial adhesins, glycosidases and antigenic similarity (Chow & Lee, 2006; Gaskins *et al.,* 2008; Walter, 2008). Therefore due to the mucin-mediated individuality of the proximal gut microbiota, further comparative ecological analyses of pigs are needed to determine interindividual variations in the abundance of *O. umbonata* and *V. magna* (Zoetendal *et al.,* 2006). Generally, the results of such comparative ecological studies have implications for an understanding of the observed high variation in the efficacy of probiotic bacteria in individual pigs (Mackie *et al.,* 1999; Nousiainen *et al.,* 2004; Simon *et al.*, 2004).

2.2 Phenotypic characteristics of indigeneity

O. umbonata and *V. magna* were both isolated from the pig mucosal jejunum. The intimate association of bacteria with gut epithelia is *per se* a criterion of indigeneity, but still, mucosa-associated isolates should not be judged *a priori* to be indigenous (Savage, 1977). Therefore I carried out phenotypic analyses of some ecologically relevant characteristics, namely oxygen relationship, production and degradation of hydrogen peroxide, growth on mucin from porcine stomach, temperature and pH range for growth and, with strains of *O. umbonata*, also bile resistance (Chapters V and VI). I have concluded from the results of my taxonomic studies that *O. umbonata* and *V. magna* are principally well adapted and presumably indigenous to the guts of pigs and other homeothermic vertebrates (Chapters V and VII).

In the following, I will discuss in more detail the phenotypic characteristics microaerotolerance, hydrogen peroxide accumulation and mucin utilisation. I choose this focus, firstly, since these characteristics are determinant in the pig mucosal jejunum and, secondly, since they presented the basis for the development and application of LHP and PGM-plus/PGM media (Chapter II).

2.2.1 Microaerotolerance

The strains of *O. umbonata* and *V. magna* were characterised as being moderately obligate (microaerotolerant) anaerobes due to their good growth in URAS (unreduced aerobically sterilised) liquid media (Chapters V and VI). The two novel species belong to the great group of obligately anaerobic gut bacteria, which are defined by their general inability to produce growth under room atmospheric (aerobic) conditions and use oxygen as electron acceptor (Savage, 1977; Richards *et al.,* 2005; Winn *et al.,* 2005; Brioukhanov & Netrusov, 2007).

Microaerotolerant anaerobes are bacteria that still grow routinely when exposed to unreduced microaerobic [i.e. oxygen-reduced (>0.5-8 % O_2, by vol.)] conditions (Loesche, 1969; Winn *et al.,* 2005). As such, they differ from the other subgroup of obligate anaerobes, the strictly anaerobic bacteria, which are already inhibited by oxygen levels greater than 0.5 % (Loesche, 1969). I did not determine the oxygen level in uninoculated URAS media due to a lack of technical means. However, using a comparable cultural approach, Lai & Chu (2008) found that the oxygen level in an uninoculated tube medium for conditional aerobic growth of the anaerobic bacterium *Treponema denticola* was 3.9 %. Therefore and based on the inability of *O. umbonata* and *V. magna* to produce normal growth on microaerobically [i.e. under oxygen-reduced (5-7 % O_2, by vol.) conditions] incubated agar media, it can be concluded that the novel species are microaerotolerant anaerobes, which are capable of growth in 'microoxic habitats' (Unden, 1999) with oxygen levels between more than 0.5 and approximately 5 %.

The isolated community of Chapter IV did not include any strictly anaerobic bacteria. This result might be an artefact due the absence of anaerobic methods for isolation, however, it is consistent with the microoxic conditions of the mucosa-associated GI microhabitats (He *et al.,* 1999; Isolauri *et al.,* 2004; Wilson, 2005) and with the general fact that 'nearly all anaerobes known to date are relatively aerotolerant, which contributes to their survival under adverse microaerobic conditions' (Brioukhanov & Netrusov, 2007). Also in pigs, strictly anaerobic bacteria are presumably restricted to the markedly hypoxic conditions of the luminal large intestine (He *et al.,* 1999).

2.2.1.1 Microaerotolerance of *O. umbonata*

Growth of the strains of *O. umbonata* was slightly impaired under unreduced microaerobic compared with reduced anaerobic culture conditions. This effect on growth rate was more pronounced during the lag and early exponential phase than during the late exponential phase (unpresented data). Hence, it might be presumed that the microaerotolerance of *O. umbonata* is mediated to some extent by self-produced lactic acid and certain free amino acids. Lactic acid is a reducing agent that decreases the redox potential (Brioukhanov & Netrusov, 2007). Free amino acids, such as arginine, may act as nutritional antioxidants and neutralise reactive oxygen species (Fang *et al.,* 2002; Jakubovics *et al.,* 2008). Unreduced microaerobic conditions caused a decrease in the formation of formic and acetic acids, whereas the major production of lactic acid remained unchanged (Chapters V and VII). I concluded that *O. umbonata* produces formic and acetic acids via the anaerobic pyruvate-formate lyase, primarily because this enzyme system is extremely sensitive to oxygen, whereas the competing lactate dehydrogenase (LDH) for the reduction of pyruvate to lactic acid is not

(Condon, 1987; Axelsson, 2004). The inhibition of the ATP-generating pyruvate-formate lyase system in URAS liquid media likely accounted for the observed reduction of growth rate. In contrast to the facultatively or aerotolerant anaerobic LAB *sensu stricto* and some bifidobacteria (Axelsson, 2004), strains of *O. umbonata* were apparently incapable of utilising oxygen as an alternative electron acceptor via flavoprotein oxidases. Such oxygen utilisation would have been apparent under microaerobic conditions by a change of sugar (glycolytic pyruvate) metabolism from lactic to acetic acid production (due to the reoxidation of NADH via the oxidases instead of LDH) and by a concomitantly increased growth rate (due to an enhanced energy yield through generation of an additional ATP from acetyl phosphate) (Condon, 1987; Vandevoorde *et al.,* 1992; von Ah *et al.,* 2007; Hammes & Hertel, 2009).

2.2.1.2 Microaerotolerance of *V. magna*

Oxygen relationship of veillonellae has long been a matter of discussion. Proposing the genus *Veillonella* in 1933, Prévot already stated that its members are strictly anaerobic but sometimes microaerophilic and able to utilise oxygen at reduced levels in liquid cultures (Prévot, 1933). Nowadays it is believed that veillonellae are strict to obligate anaerobes with a principally fermentative type of metabolism that may grow aerobically in static culture on lactic acid (Holt *et al.*, 1994; Kolenbrander, 2006; Carlier, 2009). Against this background, it is not surprising that *V. magna* lac18^T was characterised as being moderately obligate (microaerotolerant) anaerobic. In comparison to *O. umbonata*, the species was less sensitive to oxygen. Strain lac18^T was capable of weak growth and hydrogen peroxide production under microaerobic [i.e. oxygen-reduced (5-7 % O_2, by vol.)] conditions (Chapter VI). It is tempting to hypothesize that veillonellae exhibit greater tolerance to oxygen due to their intraphylum co-evolution with the facultatively or aerotolerant anaerobic LAB *sensu stricto*. Microaerotolerance of *V. magna* is presumably mediated by the reductive effects of lactic acid and hydrogen sulphide. As I have determined in the study of Chapter VI, *V. magna* lac18^T is able to produce hydrogen sulphide. Hydrogen sulphide is a reducing agent and antioxidant scavenger of hydrogen peroxide (Krieg & Hoffmann, 1986; Schmitz *et al.,* 2006). It may also be involved in the self-generation of local microanaerobic environments through reductive removal of dissolved oxygen (Lai & Chu, 2008).

Anaerobic growth of *V. magna* lac18^T was noticeably better in a carbon dioxide atmosphere (18 % CO_2, by vol.) than in the same nitrogen-purged medium (approx. 100 % N_2, by vol.) (unpresented data). This fits with the assumption that carbon dioxide is required and often stimulatory to the growth of veillonellae (Rogosa, 1964; Holt *et al.*, 1994). Veillonellae are not auxotroph for carbon dioxide, since they produce it in lactic acid fermentation (Delwiche *et al.,* 1985; Holt *et al.*, 1994). *V. magna* lac18^T was found positive for production of gas,

probably carbon dioxide, hydrogen and potentially hydrogen sulphide (Delwiche *et al.,* 1985; Holt *et al.*, 1994; Seeliger *et al.,* 2002; Chapter V). In the pig duodenum and proximal jejunum, carbon dioxide is inherently present in the mucosa-associated microhabitats from the reaction of secreted bicarbonate from Brünner's glands with hydrogen ions in mucus (Moran, 1982; Bacha & Bacha, 2000; Breves *et al.,* 2000). It is also provided in the small intestine by other bacteria, above all co-colonising heterofermentative lactobacilli and other LAB *sensu stricto* (Axelsson, 2004).

2.2.1.3 Microaerotolerance in mucosal bacterial communities

In the pig small intestine, lactic acid-related bacteria (LARB) colonise the mucosa-associated microhabitats in form of microcolony communities (Chapter II). It is believed that mucosa-associated communities are not randomly but orderly structured. They probably exhibit distinct patterns of spatial organisation that are determined by two main physicochemical gradients in the inner mucus gel layer: primarily the viscosity and secondarily the oxygen gradient (Chow & Lee, 2006; Swidsinski *et al.,* 2007). *O. umbonata* and *V. magna* are both non-motile and relatively short and small bacteria. It can be assumed that strains of these species are restricted to the outer mucosal sites, since due to their cell morphotypes, they are less able to penetrate the highly viscous mucus layer adjacent to the absorptive epithelia or to withstand the excretory flow in the mucosal crypts (Lee, 1985; Swidsinski *et al.,* 2007). The residence of these obligately anaerobic bacteria in the outer mucosal sites is plausible, in so far as the oxygen level is certainly lower in the mucus zone closer to the intestinal lumen than at the epithelia (Wilson, 2005).

The intramucus oxygen gradient is sustained through consumption of oxygen by facultatively or aerotolerant anaerobic bacteria that, enabled by their long, rod-shaped and occasionally motile morphotype, are situated within the mucosa-adjacent mucus layer (Swidsinski *et al.,* 2007). With regard to the isolated community of Chapter IV, this ecological niche was presumably inhabited by strains of *Escherichia coli*, *Bacillus* sp. and *L. agilis*, most of the latter exhibiting vigorous motility in a self-produced, highly viscous extracellular polymeric substance (unpresented data). The generation of a reduced, microoxic environment by initially colonising oxidase-positive species is a major autogenic factor of succession during the development of adult microbiota patterns in the gut (Nousiainen *et al.,* 2004; Wilson, 2005; Chow & Lee, 2006; Gaskins *et al.,* 2008).

Spatial organisation of bacterial communities generally promotes beneficial interrelationships and adaptive responses, among others, through intra- and interspecies quorum sensing

(Wilson, 2005; Camp *et al.,* 2009). Mucosal microcolony and biofilm communities of LARB thus facilitate physical (coaggregative), nutritional (syntrophic) and genomic (horizontal gene transfer) interactions between constituent members. Both cellular coaggregation and lactic acid syntrophism are involved in the local generation of micro- or anaerobic, low redox environments for the protection of obligately anaerobic species (Brioukhanov & Netrusov, 2007; Jakubovics *et al.,* 2008; Ledder *et al.,* 2008). Hence, in the pig small intestine, microcolony communities of LARB likely present a range of microhabitats and niches (Wilson, 2005) for phenotypically diverse species. Mucosa-associated indigenous bacteria generally stimulate epithelial angiogenesis and oxygen leakage from the gut microcirculation (Hooper *et al.,* 2001; Stappenbeck *et al.,* 2002; Zoetendal *et al.,* 2006; Leser & Mølbak, 2009). They thus benefit the host through facilitating increased ecological diversity and stability (protective benefit) and absorptive capacity (nutritional benefit). Interestingly, it has been found that short-chain fatty acids (SCFA) from the fermentative metabolism of anaerobic bacteria may counteract this effect (Ogawa & Binion, 2005). It remains to suppose that *O. umbonata* and *V. magna* exert such a counteractive effect *in vivo*.

2.2.2 Hydrogen peroxide accumulation

I analysed hydrogen peroxide production and degradation by *O. umbonata* and *V. magna* (Chapters V and VI), since these characteristics are integral parts of anti-oxidative defence and ecological balancing of communities of LARB in microaerobic habitats (Condon, 1987; van de Guchte *et al.,* 2002; Rochat *et al.,* 2006; Jakubovics *et al.,* 2008).

Hydrogen peroxide production has been an overlooked characteristic in the descriptions of olsenellae and veillonellae (Dewhirst *et al.,* 2001; Holt *et al.*, 1994; Carlier, 2009). Strains of *O. umbonata* did not produce noticeable amounts of hydrogen peroxide, and also *V. magna* lac18^T exhibited only weak and moderate accumulation under anaerobic and microaerobic culture conditions, respectively (Chapters V and VI). These results are not surprising given the fact that both species are obligate anaerobes with a principally not oxygen-reductive but fermentative metabolism. It can be assumed that hydrogen peroxide was produced in *V. magna* lac18^T by superoxide reductase and/or superoxide dismutase. Superoxide reductase and dismutase are the frontline oxidative defence enzymes in moderately obligate anaerobic and microaerophilic bacteria (Rolfe *et al.,* 1978; Kurtz, 2004; Winn *et al.,* 2005; Brioukhanov & Netrusov, 2007; Imlay, 2008; Madigan *et al.,* 2008). They are indispensable due to the formation of reactive oxygen species by growing cultures even under anaerobic conditions (Brioukhanov & Netrusov, 2007).

The ecological implications of these results are debatable. On the one hand, production of hydrogen peroxide is often considered a probiotic feature, because it confers a protective benefit to the host by interbacterial amensalism (interference competition) (Annuk *et al.,* 2003; Nousiainen *et al.,* 2004; Ouwehand & Vesterlund, 2004; Wilson, 2005). This is especially relevant in communities of LARB, since it was found that hydrogen peroxide can act synergistically with lactic acid in the inhibition of enteric pathogens (Atassi & Servin, 2010). However, on the other hand, bacterial hydrogen peroxide is a potential irritant also to host gut epithelial cells, and there seem to be increasing reports of host-directed detrimental effects (Zalán *et al.,* 2005; Koller *et al.,* 2008; Strus *et al.,* 2009).

Degradation of hydrogen peroxide in the catalase test was negative for strains of *O. umbonata* and negative or unspecifically positive (delayed and weak) for *V. magna* lac18^T (Chapters V and VI). The unspecific reaction of *V. magna* was probably due to an atypical catalase lacking porphyrin (haem) as has been observed in other veillonellae and LAB *sensu stricto* (Rogosa, 1964; Holt *et al.*, 1994; Carlier, 2009; reviewed in Chapter III). It was to be expected that the two novel species are negative for the true catalase, since this enzyme is generally missing in anaerobic bacteria (Percy, 1984; Schmitz *et al.,* 2006). Nevertheless, as other obligately anaerobic gut bacteria, strains of both species are necessarily equipped with other enzymatic or non-enzymatic systems of hydrogen peroxide defence. Besides the atypical catalase, these probably include peroxidase-like enzymes and, in the case of *V. magna*, also hydrogen sulphide (Krieg & Hoffmann, 1986; Brioukhanov & Netrusov, 2007; Imlay, 2008; Madigan *et al.,* 2008).

Hydrogen peroxide is invariably present in the mucus layer of the pig proximal gut, not only from microbial oxygen reduction reactions, but also as an integral component of the host-derived peroxidase-thiocyanate-hydrogen peroxide system (Allaoui *et al.,* 2009). Linked with the co-evolution of mammals and their predominantly Gram-stain-positive gut bacteria, this system presents a major innate immune defence mechanism with antibacterial selectivity against Gram-stain-negative bacteria, thus promoting a homeostatic ecosystem (Reiter *et al.,* 1980; Reiter & Härnulv, 1984; Vandevoorde *et al.,* 1992; Ouwehand & Vesterlund, 2004). The susceptibility to this system of *V. magna* and other veillonellae of the *Negativicutes* is questionable. The peroxidase-thiocyanate-hydrogen peroxide system is traditionally thought to act bactericidal against Gram-stain-negative bacteria through inhibition of cytoplasmic membrane functions and several reactions of glycolysis (Vandevoorde *et al.,* 1992; Ouwehand & Vesterlund, 2004; Hammes & Hertel, 2009). Veillonellae might be less susceptible than for example *E. coli* or salmonellae, since they are metabolically independent of the glycolytic pathway. They may also counteract the pH-dependent effect of the system

(Reiter & Härnulv, 1984) by conversion of lactic acid (pK_a=3.9) into less acidic propionic (pK_a=4.9) and acetic (pK_a=4.8) acids (Partanen & Mroz, 1999; Wikipedia, 2010). *V. magna* lac18^T has been found to exert such an alkalinising effect at low pH values (Chapter VI). However, what seems most important is that, according to latest results (Allaoui *et al.,* 2009), the components of the system are diluted in the GI mucus gel matrix and, therefore are presumably active as mere chemorepellents rather than as bactericids. Furthermore, coaggregation of veillonellae and LAB in mixed biofilm or microcolony communities may multiply their potential for hydrogen peroxide defence (Kondo & Onisi, 1962; Allaoui *et al.,* 2009).

2.2.3 Mucin utilisation

O. umbonata and *V. magna* are both capable of growth in pure culture on porcine gastric mucin (PGM) as sole carbon and nitrogen source. Strains of *O. umbonata* exhibited moderate to good growth and physiological cell morphotypes on a PGM agar medium (Chapter V). Using the techniques of de Ferro *et al.* (1999), growth of *V. magna* lac18^T was visibly enhanced by the spent supernatants of *Enterococcus* isolate lac32 or the probiotic strain *Enterococcus faecium* NCIMB 10415, almost certainly due to the presence of lactic acid (unpresented data). This observation might indicate that *V. magna* lac18^T is able to utilise mucins as nitrogen rather than as carbon and energy source. Putrescine, if also produced by the enterococci, might have exerted an additional positive effect on *V. magna* lac18^T. The two novel species were not growing strongly on PGM and therefore do not seem to qualify as mucin oligosaccharide degrading bacteria (Hoskins, 1992). This group comprises about one to five percent of the cultivable bacteria in human faeces and, hence apparently, is a rather small, functionally distinct subset of the indigenous gut microbiota (Hoskins, 1992; Chassard *et al.,* 2008).

Mucin utilisation is a prior criterion of colonisation ability and thus indigeneity to the proximal GI tract (Lee, 1985; Goméz, 2006; Krüger *et al.,* 2008; Fakhry *et al.,* 2009). Genes for mucin glycoprotein degradation and fermentation present 'niche-specific genes' (Walter, 2005) of ecologically specialised and highly adapted species (Walter, 2008). Some of these genes, such as for *N*-acetyl-D-glucosamine fermentation in lactobacilli, are plasmid-encoded and might therefore be subject to horizontal gene transfer within related genera of *Firmicutes* (Hammes & Hertel, 2006). Since there are strong barriers toward interphylum horizontal gene transfer (Azcarate-Peril *et al.,* 2008), it can be assumed that strains of *O. umbonata* are excluded from the benefits of such genetic interrelationships in the predominantly *Firmicutes*-containing communities of the pig small intestine. However, *O. umbonata* exhibits a protease and a wide range of exopeptidases (Chapters V and VII), which might be involved in the

degradation of tertiary mucin structures and hence in reduction of mucus viscosity (Lee, 1985; Hoskins, 1992). A local microenvironment with a reduced mucus viscosity facilitates colonisation, especially for bacteria with a non-motile, non-spiral-shaped morphotype (Lee, 1985), such as the strains of *O. umbonata*.

The ability of gut bacteria to utilise mucins is a controversial property concerning the host-microbiota relationship (Fakhry *et al.,* 2009). The mucus gel layers are important components of the innate host gut defence system (Wilson, 2005), and therefore significant degradation of mucins and mucus is considered as indicative of a disadvantageous, possibly pathogenic invasive potential (Hammes & Hertel, 2006; Barbés, 2008; Fakhry *et al.,* 2009). Furthermore, massive bacterial mucinolysis necessitates compensation through enhanced mucus production, which imposes a high expenditure of metabolic energy on the pig host (Richards *et al.,* 2005). In the other extreme, in germfree animals, the absence of specific bacterial glycosidases prevents regular mucin turnover and leads to unfavourable dilatative accumulation of mucins in the ileum and large intestine (Collinder *et al.,* 2003; Pai & Kang, 2008). Besides this, mucins present the main endogenous substrates available to the gut microbiota, and their complete microbial utilisation benefits the host by the recycling of nitrogen and energy in form of acetate and other SCFA (Collinder *et al.,* 2003; Wilson, 2005; Chassard *et al.,* 2008). Therefore overall, it is most beneficial if strains of an individual bacterial species contribute to a balanced equilibrium between normal endogenous secretion and microbial utilisation of mucins and mucus (Collinder *et al.,* 2003; Fakhry *et al.,* 2009). Such a balancing effect is considered probiotic in light of the associated ability of bacteria to colonise the mucus layer and exert effects of competitive exclusion (Nousiainen *et al.,* 2004; Il Kim *et al.,* 2007; Lebeer *et al.,* 2008). So far as it can be concluded from the results of my studies, *O. umbonata* and *V. magna* seem to fit in this category of beneficial gut bacteria.

3. Ecological niches and host-relationship

The conclusion that *O. umbonata* and *V. magna* are indigenous to the pig GI tract necessarily implies that these species inhabit a particular metabolic site (niche) by exerting a demonstrable ecological function (Tannock, 2005a). In the following, I will discuss aspects of their supposed niches in the pig small intestine during digestive and interdigestive periods. Eventually, I will deduce from the ‘inhabited functions’ the relationship these species are likely to have with the pig host. The ecological relationship of individual indigenous species is debatable, in so far as, according to Tannock (2005a), the vast majority of members of the mutualistic microbiota are commensals, if considered on their own.

3.1 *O. umbonata* and metabolism of exogenous carbohydrates

My strains of *O. umbonata* were positive for fermentation of six out of 18 carbohydrates, namely the mono- and disaccharides glucose, mannose, maltose, fructose, saccharose and trehalose (Chapters V and VII). The strains of this species consistently exhibited a highly active *α*-glucosidase, the only positive sugar hydrolase using three different API kits (Chapter V). Especially glucose and fructose are ubiquitous and abundant substrates in the digestive mucosal jejunum of pigs (reviewed in Chapter II). They present readily absorbable monomer sugars that can be efficiently shunted into the glycolytic Embden-Meyerhof-Parnas pathway (Turroni *et al.,* 2008). The observed sugar utilisation pattern thus indicates that *O. umbonata*, as many fastidious and auxotroph intestinal lactobacilli, has evolved an adaptive, niche-specific glycobiome conferring competitive fitness in the nutrient-rich habitats of the pig small intestine (Azcarate-Peril *et al.,* 2008; Lebeer *et al.,* 2008; Hammes & Hertel, 2009; Kleerebezem *et al.,* 2010).

The strains of *O. umbonata* are presumably obligately homofermentative, since they were unable to ferment pentose sugars or produce noticeable amounts of gas (Axelsson, 2004; Chapter V). Hence, as bifidobacteria and in contrast to some lactobacilli (Simon *et al.,* 2005), they are not supported by arabinose or xylose that may be released in the pig small intestine from antinutritive arabinoxylans (pentosans) by exogenously supplemented xylanases. The growth of all strains of *O. umbonata* was vigorous using fructose as sole carbon and energy source (Chapter V, unpresented data). This result might indicate that the species is able to utilise fructose as electron acceptor in anaerobic respiration of fructose, and possibly also glucose and maltose (Axelsson, 2004). However, the species was unable to grow on inulin, the polymer of *ß*-linked fructose (Chapter V). Dietary inulin is considered to exert beneficial effects on the host, above all by prebiotic stimulation of populations of bifidobacteria (Simon *et al.*, 2004; Niba *et al.,* 2009). Saccharose fermentation by *O. umbonata* is ecologically relevant, in that some lactobacilli, such as strains of *L. reuteri*, produce saccharose-containing exopolysaccharides, which are suggested to exert a syntrophic effect on co-colonising bacteria, especially during conditions of exogenous carbohydrate depletion (Reuter, 2007; Lebeer *et al.,* 2008). As typical for LAB (Orla-Jensen, 1919), the strains of *O. umbonata* did not ferment galactose (Chapter V). They exhibited no active *ß*-galactosidase and consequently were unable to utilise lactose (Chapter V). Considering that lactose presents the predominant sugar in sow's milk, these results imply that *O. umbonata* does not belong to the very early colonisers of the pig GI tract. This conclusion is plausible, in so far as very early colonisation would probably necessitate higher oxygen tolerance. Other *ß*-glycosidases, namely *ß*-glucosidase and *ß*-glucuronidase, were also negative in all strains of *O. umbonata* (Chapter V). Lacking *ß*-glucosidase, the species is unable to degrade intact complex plant cell wall

polysaccharides, i.e. cellulose, 1-3,1-4-*ß*-glucans, xyloglucans and other antinutritive glucose-containing hemicelluloses (Simon, 2008). Therefore it may not contribute to more proximal bacterial utilisation of these non-starch polysaccharides and thus enhanced energy harvest by the host (Niba *et al.,* 2009). *O. umbonata* qualifies as a 'non-fiber digester', however strains of this species could be competitive enough to benefit from 'primary substrate leakage' (Lee, 1985) in co-colonisation with interrelated cellulolytic and hemicellulolytic bacteria, such as some other LAB and *Bacteroides* spp., in the pig large intestine (Kenworthy, 1973; Goméz, 2006; Walter, 2008). From a more directly health-related point of view, the absence of *ß*-glycosidases (*ß*-galactosidase, *ß*-glucosidase and *ß*-glucuronidase) in *O. umbonata* can be regarded as beneficial, as these enzymes have been implicated in the formation of toxins, carcinogens and mutagens in the human gut (Sandine, 1979; Wilson, 2005; Goldin, 2006). However, it is conceivable that the species is subject to interbacterial induction of *ß*-glycosidases and possibly other genes for carbohydrate metabolism in multispecies communities *in vivo* (Azcarate-Peril *et al.,* 2008; Turroni *et al.,* 2008).

3.1.1 *O. umbonata* and SCFA from carbohydrates

As other olsenellae, *O. umbonata* belongs to the group of LAB with lactic acid constituting the major metabolic product from glucose. Besides lactic acid, the species produced minor amounts of two volatile SCFA, namely formic and acetic acids (Chapters V and VII). SCFA are the main metabolites of bacterial carbohydrate fermentation in the gut (Gancarčiková *et al.*, 2009). They are known to play a crucial role in gut health and environment (Chassard *et al.,* 2008). A vast array of physiological, clinical and ecological functions is attributed to SCFA, making them an extremely interesting parameter (Collinder *et al.,* 2003). Some SCFA, including lactic, formic, acetic and propionic acids, are used as health-beneficent and growth-promoting feed additives for pigs, first of all in the nutrition of newly weaned pigs (Partanen & Mroz, 1999; Richards *et al.,* 2005; Simon, 2008).

3.1.1.1 Effects of bacterial SCFA

Firstly, SCFA exert important local effects on gut morphology and physiology. It is generally assumed that SCFA stimulate epithelial cell growth and immunity as well as regulate the underlying endothelial microvasculature (Ogawa & Binion, 2005; Richards *et al.,* 2005; Norin & Midtvedt, 2006; Gancarčiková *et al.*, 2009; Niba *et al.,* 2009). SCFA also impact on gut physiology by promoting peristalsis and motility (Walter, 2005; Goméz, 2006; Hammes & Hertel, 2006; Pai & Kang, 2008). Hence overall, SCFA play a key role in maintaining gut mucosal homeostasis and barrier function (Ogawa & Binion, 2005).

Secondly, serving as bacterial 'carbon release valve' (Kai *et al.,* 2009), SCFA present an energy supply for epithelial gut cells and also the host systemic metabolism (Wilson, 2005; Norin & Midtvedt, 2006). Depending on the individual acid, they are involved in different systemic metabolic pathways (Partanen & Mroz, 1999; Goldin, 2006; Norin & Midtvedt, 2006). SCFA are rapidly absorbed from the pig stomach and small intestine (Kenworthy, 1973; Tannock, 1990; Collinder *et al.,* 2003). However, as they contain only about 60-75 % of the energy content of ingested carbohydrates, their production in the pig proximal GI tract is associated with some energy loss to the host (Hooper *et al.,* 2002; Niba *et al.,* 2009). SCFA have been shown to facilitate the absorption of positively charged dietary minerals (calcium, phosphorus, magnesium and zinc) in pigs and other mammals, *inter alia*, by induction of the intestinal epithelial vitamin D receptor (Allen & Snary, 1972; Kenworthy, 1973; Hismiogullari *et al.,* 2008; Resta, 2009). They may improve the praecaecal digestibility of protein and other nutrients through an elevation of gastric proteolysis, pancreatic secretions and absorptive surface area (Richards *et al.,* 2005; Apajalahti *et al.,* 2009; Niba *et al.,* 2009).

Thirdly, SCFA function as 'bioactive infochemicals' in the development and sustainment of cooperative bacterial communities (Kai *et al.,* 2009). Their ecological impact in the gut is of a dual nature, in so far as they enhance the growth of some specific microbes (e.g. lactic acid stimulates veillonellae and megasphaerae), while reducing the overall community diversity through inhibition of others, such as undesirable, potentially pathogenic Gram-stain-negative bacteria and clostridia (Sandine, 1979; Nousiainen *et al.,* 2004; Richards *et al.,* 2005; Norin & Midtvedt, 2006; Kai *et al.,* 2009). Lactic, acetic and propionic acids are the most common inhibitory primary metabolites of competing LARB in highly nutrient rich habitats, such as the pig proximal gut (Ouwehand & Vesterlund, 2004; Nousiainen *et al.,* 2004). They contribute to the formation of an 'acid barrier' (Tannock, 1990) in the stomach and mucosal jejunum of pigs. This barrier is an important part of non-specific colonisation and disease resistance mechanisms, especially in the weakly hydrochloric stomach of newborn piglets (Lee, 1985; Pedersen & Tannock, 1989; Nousiainen *et al.,* 2004; Bomba *et al.,* 2006; Rodehutscord, 2008).

3.1.1.2 Inhibitory mode of action of bacterial SCFA

The ecological principal behind the action of SCFA in the gut is interference competition or amensalism: a fastidious, acid-producing bacterium gains advantage over another competing microbe in a nutrient rich habitat by changing the habitat environment through external acidification and chelative capture of essential elements, such as iron for facultatively anaerobic, cytochrome-containing bacteria, and by exerting internal detrimental effects

(Vandevoorde *et al.,* 1992; Ouwehand & Vesterlund, 2004; Wilson, 2005; Lebeer *et al.,* 2008).

External reduction of pH (external acidification) is considered to be *per se* an effect of bacterial SCFA. SCFA are weak organic acids, and at lower pH they are present rather in the undissociated form, which is better diffusible across bacterial cell walls and internally most destructive (Sandine, 1979; Ouwehand & Vesterlund, 2004; Hammes & Hertel, 2009). The relevance of external acidification in the pig small intestine is debatable, in so far as pancreatic secretions may neutralise this effect of SCFA (Apajalahti *et al.,* 2009).

The precise internal actions of acid stress on bacterial physiology are not fully known (Lebeer *et al.,* 2008). Traditionally, the antibacterial action of SCFA was explained by the effects of cytoplasmic acidification on acid-sensitive enzymes resulting in reduced energy yield and/or damage to cellular macromolecules (Russell, 1992). Besides this, cytoplasmic acidification enhances oxidative stress through promotion of the hydroxyl radical-generating Fenton reaction (Imlay, 2008). More recently it has been recognised by the so-called uncoupling or chemiosmotic theory that the internal dissociation of SCFA causes an energy-expending dissipation of the transmembrane electrochemical gradient (protonmotive force) as well as an accumulation of anions in the cytoplasm (Russell, 1992; Ouwehand & Vesterlund, 2004; Lebeer *et al.,* 2008; Carpenter & Broadbent, 2009). Cytoplasmic anion accumulation is supposed to be the primary inhibitory mode of action of SCFA due to an increase of cellular osmolarity and turgor pressure as well as inhibition of transmembrane transport, macromolecular synthetic and other important metabolic processes (Ouwehand & Vesterlund, 2004; Hismiogullari *et al.,* 2008; Carpenter & Broadbent, 2009). Bacteria can counteract the negative effects of anion accumulation by lowering the internal pH and thus the internal:external pH gradient and by increasing anion efflux (Ouwehand & Vesterlund, 2004; Hismiogullari *et al.,* 2008). The specific efficacy of SCFA against Gram-stain-negative bacteria is based on the greater resilience of the Gram-stain-positive type of cell wall and on the greater capability of Gram-stain-positive bacteria to reduce the internal pH as a function of the external pH (Russell, 1992; Hismiogullari *et al.,* 2008). In accordance with this, the limit of the pH range for growth was lower for the strains of *O. umbonata* (pH 4.5) than for *V. magna* lac18^{T} (pH 5.5) (Chapters V and VI). However, *O. umbonata* might be less acid-tolerant than other LAB due to the pH-sensitivity of the pyruvate-formate lyase system (Russell, 1992).

3.1.1.3 *O. umbonata* and D-lactic and formic acids

The strains of *O. umbonata* produced major amounts of D-lactic acid under both anaerobic and microaerobic conditions. They did not produce L-lactic acid (Chapters V and VII). The D- or L-configuration of lactic acid depends on the stereospecificity of the LDH enzyme and is a species-specific characteristic (Orla-Jensen, 1919; Hammes & Hertel, 2006). The production of only the D-lactic acid enantiomer is shared by heterofermentative leuconostocs (Axelsson, 2004) and, so far known, is a rather rare feature among LAB in the gut. It complements the production of only L-lactic acid by bifidobacteria (Belenguer *et al.,* 2007). The antimicrobial action of lactic acid is directed primarily against bacteria (Partanen & Mroz, 1999). D-lactic acid may display a greater or lower antibacterial activity than L- or DL-lactic acids, depending on the external pH condition and the targeted species (Fayol-Messaoudi *et al.,* 2005; Carpenter & Broadbent, 2009). Lactic acid is considered a key antimicrobial SCFA, because it reduces the external pH very effectively and acts as a permeabiliser of the outer membrane of Gram-stain-negative pathogenic bacteria (Tannock, 1992; Fayol-Messaoudi *et al.,* 2005; Hismiogullari *et al.,* 2008; Pieper *et al.,* 2008). In addition, lactic acid may indirectly promote acidification in the pig GI tract, since, in contrast to other SCFA, it does not stimulate the mucosal secretion of neutralising bicarbonate (Tsukahara & Ushida, 2001). Overall the effects of lactic acid lay the basis for the synergistic, i.e. potentiated amensalistic, efficacy of mixtures of SCFA (Vandevoorde *et al.,* 1992; Ouwehand & Vesterlund, 2004; Lebeer *et al.,* 2008).

The D-configuration of lactic acid from *O. umbonata* may be ecologically relevant in communities of LARB, firstly, in that D-lactic acid can confer enhanced vancomycin resistance to lactobacilli (Kleerebezem *et al.,* 2010) and, secondly, in that some lactic acid-utilising bacteria strongly prefer it to the L-enantiomer (Duncan *et al.,* 2004). Removal of accruing D-lactic acid is vital, since the accumulation of this acid can have severe systemic consequences for host health (Newbold *et al.,* 1987; Duncan *et al.,* 2004; Hammes & Hertel, 2006; Hammes & Hertel, 2009). Excessive production of D-lactic acid is counteracted through inhibition of the D-LDH by the host peroxidase-thiocyanate-hydrogen peroxide system (Reiter & Härnulv, 1984).

The strains of *O. umbonata* formed formic acid as a side product, most notably under anaerobic conditions (Chapters V and VII). Formic acid (pK_a=3.7) is even a stronger acid than lactic acid (pK_a=3.9) (Partanen & Mroz, 1999; Wikipedia, 2010), so it is likely that small amounts contribute effectively to acidification in the gut. However, the production of greater amounts of basic ammonia (pK_a=9.3) (Wikipedia, 2010) by *O. umbonata* (Chapters V and VII) presumably exerts a local buffering effect in view of its formic acid. Formic acid is one

of the most commonly used organic acids as dietary acidifiers for pigs, and high concentrations have been shown to exert positive inhibitory effects on the pig small intestinal microbiota (Partanen & Mroz, 1999; Apajalahti et al., 2009). However, it must be assumed from the results of Apajalahti *et al.* (2009) that the production of formic acid by *O. umbonata* and other bacteria *in vivo* normally leads to only low concentrations that rather stimulate than inhibit the intestinal microbiota.

3.2 *O. umbonata* and metabolism of exogenous organic nitrogen compounds

The strains of *O. umbonata* were found positive for a gelatinolytic protease, two dipeptidyl peptidases and 12 out of 14 other exopeptidases (Eschenlauer *et al.*, 2002; Chapters V and VII). This marked activity on organic nitrogen compounds is noteworthy, in that lactobacilli and other LAB *sensu stricto* are characteristically negative for gelatinolytic protease and poorly quipped with other enzymes involved in the degradation of proteins, peptides and amino acids (Orla-Jensen, 1919; Barbés, 2008; Hammes & Hertel, 2009). It is also therefore generally assumed that bacterial communities contribute rather insignificantly to proteolysis in the pig proximal gut (Nousiainen *et al.,* 2004; Goméz, 2006).

Proteases and peptidases of gut bacteria impact on the host intestinal nitrogen recycling and systemic nitrogen balance in multiple ways (Pai & Kang, 2008; Resta, 2009). Some of these impacts are beneficial to the host. In the pig small intestine, proteolytic and peptidolytic bacteria may degrade pancreatic enzymes (e.g. trypsin) and/or dietary inhibitory peptides (e.g. trypsin inactivators in soy extraction grist (Tannock, 1995a; Collinder *et al.,* 2003; Norin & Midtvedt, 2006; Simon, 2008). Trypsin utilisation of bacteria is viewed positively, in so far as a lower excretion of tryptic activity in faeces represents a smaller energy loss to the host (Collinder *et al.,* 2003). Secondly, bacteria with proteases and peptidases may improve the host nitrogen balance by converting endogenous nitrogen compounds that are expelled with enterocytes (e.g. cell-surface mucins) into absorbable compounds (Norin & Midtvedt, 2006). Thirdly, it is well established that proteolytic and peptidolytic LAB liberate specific peptides and amino acids that stimulate co-existing species, such as veillonellae and/or lactobacilli and other LAB with positive arginine deiminase pathway (van de Guchte *et al.,* 2001; Axelsson, 2004; Barbés, 2008; Crippen *et al.,* 2008; Hammes & Hertel, 2009). Hence, some bacterial peptide breakdown products in faeces serve as indicator of intestinal colonisation resistance due to enhanced community diversity and activity (Collinder *et al.,* 2003). Strains of *O. umbonata* were arginine deiminase (dihydrolase) positive, however not stimulated by L-arginine in the presence of glucose and are therefore apparently negative for the ATP-generating reaction of the arginine deiminase pathway (Zúñiga *et al.*, 2002; Axelsson, 2004; Chapter V).

3.2.1 *O. umbonata* and ammonia from peptides and amino acids

Bacterial proteases are generally considered to be involved in pathogenicity (Bascomb, 1987; Wilson, 2005). Since the work of Metchnikoff (1845-1916) on LAB and longevity, the deleterious effects of 'putrefactive' gut bacteria have been related to the production of toxic nitrogen catabolites, above all ammonia and amines (Nousiainen *et al.,* 2004; Walter, 2005; Leser & Mølbak, 2009). Ammonia may be a causative factor of diarrhoeal disorders and growth depression in pigs (Richards *et al.,* 2005; Niba *et al.,* 2009). Thus the performance enhancing effects of antimicrobial growth promotants in pig production have been explained by the suppression of hyper-ammonia-producing (HAP) gut bacteria (Kenworthy, 1973; Eschenlauer *et al.*, 2002; Nousiainen *et al.,* 2004; Edwards *et al.,* 2005).

Strain A2 of *O. umbonata* has been found to produce ammonia by deaminase activity on trypticase, a pancreatic casein hydrolysate containing predominantly peptides (Eschenlauer *et al.*, 2002). Gut bacteria may also form ammonia by reduction of nitrite (nitrate ammonification), hydrolysis of urea and different pathways of amino acid deamination (Wolf & Hammes, 1988; Nousiainen *et al.,* 2004; Goldin, 2006). *O. umbonata* was negative for nitrate reduction and urease, however all strains exhibited arginine dihydrolase (deiminase) activity (Chapter V). Therefore it can be assumed that the species produces ammonia predominantly by hydrolytic deamination of peptides and amino acids, such as arginine.

3.2.1.1 Effects of bacterial ammonia

The detrimental effects of ammonia on the pig host are generally twofold. Firstly, ammonia is an irritant to the GI mucosae. It causes increased gut epithelial cell turnover and impaired enzyme activity of the enterocyte brush border, which lead to enhanced endogenous losses of proteins and reduced nutrient absorption (Nousiainen *et al.,* 2004; Richards *et al.,* 2005; Goméz, 2006; Niba *et al.,* 2009). Secondly, though ammonia is principally useful in the systemic synthesis of non-essential amino acids, excessive deamination of ingested proteins and absorption of ammonia cause inefficient nitrogen retention and demand energy from the host for detoxification and enterohepatic circulation of urea (Kenworthy, 1973; Eschenlauer *et al.*, 2002; Nousiainen *et al.,* 2004). Positive effects occur, in so far as it was shown that ammonia may maintain the small intestinal mucosal system through serving in enterocytes as a precursor for the synthesis of the amino acids glutamine and glutamate, both of which present a major energy source to these cells (Moran, 1982; Breves *et al.,* 2000; Azcarate-Peril *et al.,* 2008; Resta, 2009). Furthermore, many intestinal bacteria can utilise ammonia as an important nitrogen source for protein synthesis, especially in the presence of bacterial pyruvate and SCFA (Kenworthy, 1973; Savage, 1977; Tannock, 1995; Niba *et al.,* 2009).

Hence ammonia in the proximal gut may lead to an improved systemic amino acid homeostasis of the pig host through bacterial synthesis of essential amino acids and thus proteins with a higher biological value (Püschner & Simon, 1988; Hooper *et al.,* 2002; Leser & Mølbak, 2009).

3.2.2 *O. umbonata* and niche refinement by non-HAP peptidolytic activity

The strains of *O. umbonata* produced 6-12 mM ammonium from peptone in a peptone-yeast extract-glucose medium (Chapters V and VII). Therefore the species does not qualify as an undesirable HAP bacterium. By definition, HAP bacteria are typically asaccharolytic and occupy the so-called 'HAP niche' by producing more than 40 mM ammonia (Eschenlauer *et al.*, 2002; Whitehead & Cotta, 2004). Analysis of stored manure has indicated that the group of HAP bacteria is small in the pig GI tract and outweighed by saccharolytic bacteria that produce only low amounts of ammonia (Whitehead & Cotta, 2004). It can be assumed that *O. umbonata* belongs to this second, predominant group of gut bacteria.

A high expression of peptidases and subsequent amino acid fermentation to ammonia and SCFA are likely to present adaptation factors that confer enhanced competitiveness over the saccharolytic LAB *sensu stricto* in the pig mucosal small intestine, a habitat that is rich in exogenous amino acids and di- and tripeptides during digestive periods (Lebeer *et al.,* 2008; reviewed in Chapter II). Accordingly, in the interdigestive small intestine of pigs, proteo- and peptidolytic activities in the concerted degradation and fermentation of endogenous mucin glycoproteins presumably present the ecological niche of *O. umbonata* (Chapters V and VII). This niche would be ecologically relevant, in that mucin degradation is initiated by proteolytic disruption of the tertiary structure of complex polymers at the sparsely glycosylated D-domains of the central protein backbones of neighbouring monomers (Hoskins, 1992; Wilson, 2005). Furthermore, likely mediated by interspecies quorum sensing in mucosal bacterial communities, non-specific proteolytic enzymes greatly enhance the growth on mucin of cross-fed bacteria with glycosidases (Bradshaw *et al.*, 1994; Wilson, 2005; Wickström *et al.,* 2009).

3.3 *V. magna* and metabolism of bacterial organic acids

V. magna belongs to the unusual group of lactic-acid utilising and propionic acid-producing bacteria (Kolenbrander, 2006; Chassard *et al.,* 2008; Carlier, 2009; Chapter VI). It can be expected that strains of this species ferment also pyruvate and other intermediary organic acids of the methylmalonyl-CoA pathway, namely oxaloacetate, malate and fumarate, to propionic and acetic acids (Delwiche *et al.,* 1985; Schlegel, 1992; Holt *et al.*, 1994; White,

2007; Seeliger *et al.,* 2002; Madigan *et al.,* 2008; Carlier, 2009). *V. magna* lac18^T was found negative for sugar hydrolases and acidification of carbohydrates, which is typical for the principally assacharolytic veillonellae (Holt *et al.*, 1994; Carlier, 2009; Chapters VI and VII). Veillonellae are also weakly active on organic nitrogen compounds. They are always negative for proteolytic gelatine hydrolysis and fermentation of amino acids (Carlier, 2009). In accordance with this, *V. magna* lac18^T exhibited, besides arginine dihydrolase for ammonia production, only three out of 14 exopeptidases (Chapters VI and VII). Therefore all in all and as other veillonellae, the species qualifies as a waste product utiliser that occupies the niche created by the organic acid metabolic side products of other co-existing microorganisms, pre-eminently LAB (Lee, 1985; Kolenbrander, 2006).

3.3.1 *V. magna* in a niche of lactic acid removal

Other than in the large intestine, lactic acid is the predominant organic acid from bacterial sugar fermentation in the pig proximal GI tract (Püschner & Simon, 1988; Partanen & Mroz, 1999; Tsukahara & Ushida, 2001; Collinder *et al.,* 2003; Nousiainen *et al.,* 2004; Goméz, 2006; Simon, 2008). Lactic and acetic acids account for most of bacterial SCFA in the small intestine of pigs, while propionic acid is negligible in amount (Nousiainen *et al.,* 2004). From this it can be assumed, that the niche of veillonellae, megasphaerae and possibly other existing lactic acid-fermenting and propionic acid-producing bacteria is rather small in the pig jejunum. This assumption is substantiated by the fact that veillonellae are relatively acid-sensitive and can be inhibited due to a low pH in the cause of lactic acid production, even though they are generally living in a mutualistic relationship with co-existing LAB (Kondo & Onisi, 1962; Stewart, 1992; de Ferro *et al.*, 1999; Tsukahara & Ushida, 2001; Kolenbrander, 2006; Belenguer *et al.,* 2007). The lower limit of the pH range for growth of *V. magna* lac18^T was 5.5, whereby the strain exerted a self-beneficial alkalinising effect (Chapter VI). Since the strain was isolated from the jejunum, it is obvious that its cells are generally able to tolerate low pH values during gastric passage, conceivably in a metabolically quiescent state.

The syntrophic niche of lactic acid removal may be small in the pig jejunum, nonetheless it is ecologically important. Lactic acid is rather slowly absorbed by gut epithelial cells and therefore readily accumulates (Tsukahara & Ushida, 2001; Tsukahara & Ushida, 2002). Conversion of lactic acid into other SCFA by bacteria counteracts hyper-lactate accumulation (Tsukahara & Ushida, 2001) and its negative consequences for both communities of LARB and the pig host. Firstly, a high concentration of lactic acid and external acidification reduce lactic fermentation or, more specifically, the lactic acid efflux-coupled energy recycling and hence growth of the LAB *sensu stricto* (van Dam, 1918; Orla-Jensen, 1919; Rogers & Whittier, 1928; Axelsson, 2004; Hammes & Hertel, 2006; Belenguer *et al.,* 2007;

Hismiogullari *et al.,* 2008). The removal of lactic acid from the vicinity of LAB by, *inter alia*, veillonellae promotes more efficient nutrient harvest and metabolic interactions in communities of LARB (Egland *et al.,* 2004; Lebeer *et al.,* 2008; Little *et al.,* 2008). Secondly, compared with other SCFA in the gut, lactic acid leads to enhanced mucosal acidification as well as reduced mucus secretion and absorption of electrolytes and water (Tsukahara & Ushida, 2001; Tsukahara & Ushida, 2002; Ushida *et al.,* 2009). Thus lactic acid is thought to be a causative agent of local GI diseases of pigs, namely gastric ulcers (Tannock & Smith, 1970; Moran, 1982; Breves *et al.,* 2000) and, possibly also due to a suboptimal pH for endogenous protein digestion (Simon, 2008), non-infectious, fermentative dyspepsia and diarrhoea (Tsukahara & Ushida, 2001; Tsukahara & Ushida, 2002; Ushida *et al.,* 2009). Administration of probiotic strains of *M. elsdenii* has been found to prevent hyper-lactate accumulation and dyspepsia in the large intestine of pigs (Ushida *et al.,* 2009). It is tempting to speculate that strains of *V. magna* are able to exert a similarly positive effect in the pig small intestine.

3.3.1.1 *V. magna* and propionic and acetic acids

V. magna produced considerable amounts of propionic and acetic acids under both an- and microaerobic conditions (Chapters VI and VII). Due to their higher pK_a values, propionic and acetic acids are more effective than lactic acid against enteropathogenic bacteria, yeasts and molds (Ouwehand & Vesterlund, 2004; Hismiogullari *et al.,* 2008; Gancarčiková *et al.*, 2009; Kai *et al.,* 2009). There are potentiated synergistic effects, in so far as, when a mixture of these three SCFA is present, lactic acid contributes mainly to external acidification, while propionic and acetic acids become undissociated and are the actual antimicrobial agents (Ouwehand & Vesterlund, 2004; Hismiogullari *et al.,* 2008). Propionic acid has a particular antimycotic action against molds (Partanen & Mroz, 1999; Ouwehand & Vesterlund, 2004; Hammes & Hertel, 2009). Acetic acid may be especially relevant in communities of mucosal LARB, because it has the potential to stimulate growth and/or hydrogen peroxide production of some lactobacilli, weissellae and possibly other LAB (de Man *et al.,* 1960; Sharpe, 1960; Endo *et al.,* 2009; Gao *et al.,* 2009).

Propionic and acetic acids differ from lactic acid in their fates in the host animal systemic metabolism after intestinal absorption. While L-lactic acid can be used in the liver for immediate energy generation via pyruvate, propionic acid is an important precursor for hepatic gluconeogenesis, and acetic acid is primarily taken up by peripheral tissues, such as skeletal and cardiac muscle, but can also be utilised by the brain and adipocytes for

lipogenesis (Partanen & Mroz, 1999; Goldin, 2006; Bäckhed & Crawford, 2010). This suggests that, also in pigs, propionic and acetic acids from, *inter alia*, veillonellae may have an effect on the lipid metabolism and body fat content (Leser & Mølbak, 2009; Bäckhed & Crawford, 2010).

3.4 *V. magna* and niche refinement by anaerobic sulphate reduction

V. magna lac18^T produced hydrogen sulphide from the sulphur amino acid L-cysteine (Chapter VI). As other veillonellae, the species *V. magna* belongs to the group of hydrogen sulphide-producing bacteria, which are presumably capable of energy generation through anaerobic respiration using sulphate as terminal electron acceptor (Rogosa & Bishop, 1964; Schlegel, 1992; Carlier, 2009). Hydrogen sulphide is a microbiostatic to microbicidal metabolite that complements the amensalistic effects of propionic and acetic acids (Savage, 1977a; Wilson, 2005).

The ability of *V. magna* to ferment lactic acid and also to produce hydrogen sulphide reflects a metabolic versatility in adaptation to anaerobic habitats with highly variable nutrient availability (Eschenlauer *et al.*, 2002). The relevance of bacterial sulphate reduction in the pig jejunum is questionable, since the atmospheric conditions there are not completely anaerobic. However, *V. magna* may use anaerobic sulphate reduction as a means of niche refinement both in the digestive and interdigestive pig proximal gut. In the presence of dietary nutrients, strains of this species can rely on lactic acid from the fermentation of exogenous carbohydrates and additionally form sulphide from exogenous sulphur compounds, such as glutathione, sulphur-containing amino acids and thiocyanate from rapeseed and other *Brassicaceae* vegetables (Schöne, 1993; Weißbach, 1993; Simon, 2008a; Carlier, 2009; Marquet *et al.,* 2009). During interdigestive periods, they may switch to lactic acid and sulphate from the bacterial degradation and fermentation of the oligosaccharide side chains of endogenous mucins (Bradshaw *et al.*, 1994; Marquet *et al.,* 2009). Such a continuous metabolic functioning would be ecologically relevant in the pig jejunum, in so far as the homeostasis of its complex microbial community depends on permanent occupation of all metabolic sites or niches (Krüger *et al.,* 2008). It can be assumed that sulphate reduction of *V. magna* is most active during passage of cells through the anaerobic lumen of the pig large intestine. This assumption is sustained by the observation that the sulphation of mucins is more pronounced in the pig large intestine compared with the proximal gut (Stanley *et al.,* 1983; Stanley *et al.,* 1986; Thomsson *et al.,* 1999).

4. Host-relationship in the pig jejunum

Finally, I will address the ecological relationship of *O. umbonata* and *V. magna*, in so far as it is conceivable from the discussed metabolic functions (niches) in the pig jejunum. Residence of the species in the jejunum generally implies a potentially detrimental competition with the pig host for endogenously digestible and absorbable nutrients (Lee, 1985; Hooper *et al.,* 2002; Krüger *et al.,* 2008; Leser & Mølbak, 2009). However, indigeneity of gut bacteria is always the outcome of a long-term, reciprocal process of adaptation and tolerance, so over all the colonisation of the pig jejunum by *O. umbonata* and *V. magna* is certainly a 'mixed blessing': whereas some of their activities are antagonistic and may be detrimental, other activities are necessarily beneficial (Tannock, 1990).

4.1 The digestive jejunum

O. umbonata is highly adapted to the utilisation of dietary 'fast food', i.e. simple carbohydrates and peptides from ingesta (Bomba *et al.,* 2006). During digestion, this species is able to negatively interfere with the host endogenous carbohydrate and protein metabolism. It may cause inefficient energy harvest and nitrogen utilisation through consumption of monosaccharides and amino acids at the jejunal brush border (Nousiainen *et al.,* 2004; Richards *et al.,* 2005; Niba *et al.,* 2009). In doing so, it is an antagonistic parasite. However, since the species does not contribute to hyper-ammonia production, its parasitism seems to be moderate, tending towards commensalism rather than towards direct, pathogenic antagonism. Other than *O. umbonata*, *V. magna* is a waste product utiliser and as such exerts less direct nutrient competition. The species qualifies as a commensal, whereby its conversion of lactic acid into propionic and acetic acids provides enhanced protective functions and is therefore beneficial to the host.

4.2 The interdigestive jejunum

During interdigestive periods, *O. umbonata* and *V. magna* presumably maintain their niches in the pig mucosal jejunum without exerting invasive, harmful effects. The species likely contribute to a greater diversity, metabolic complexity and hence stability of the pig jejunal ecosystem by proteo- and peptidolytic activities (*O. umbonata*) and removal of accruing lactic acid (*V. magna*) in the concerted bacterial degradation and fermentation of endogenous mucins. In this respect, they are mutualists that confer protective benefits.

The nutritive competition of *O. umbonata* during digestion presents an example of how the protective benefits imparted by the indigenous microbiota come at a toll to the pig host, even under ideal conditions (Richards *et al.,* 2005).

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Chapter IX

Summary

Kraatz, M. (2011). Isolation of lactic acid-related bacteria from the pig mucosal proximal gastrointestinal tract, including *Olsenella umbonata* sp. nov. and *Veillonella magna* sp. nov.

I decided to dedicate my thesis to the subject of the isolation of lactobacilli and other lactic acid-related bacteria (LARB) from the mucosae of the stomachs and jejuna of healthy domestic pigs, as the interactions of these indigenous microorganisms and microhabitats are utterly relevant for host animal nutrition and health.

In a first approach, I applied *Lactobacillus*-hydrogen peroxide (LHP), a newly developed culture medium for the one-step direct specific isolation and qualitative analysis of hydrogen peroxide accumulation of lactobacilli. In combination with a screening catalase-benzidine dihydrochloride test, LHP constitutes a simple approach for the detection of manganese catalase-positive isolates of potentially novel *Lactobacillus* species. The new medium was validated as highly specific and limitedly sensitive for lactobacilli. The application of LHP facilitated the hitherto unreported detection of a haem-independent manganese catalase in the type strain of *Lactobacillus plantarum* subsp. *plantarum*. However, a colony isolate with an active catalase was not detected, and hence this first approach resulted in no promisingly novel *Lactobacillus* strain.

In a second approach, I used commercial mucin from porcine stomach as basal component of two newly created culture media for the specific isolation of mucosa-associated LARB. The isolated community comprised all three groups of LARB of the *Firmicutes* and *Actinobacteria*, namely the lactic acid-producing bacteria *sensu stricto* and *sensu lato* plus the metabolically linked lactic acid-fermenting bacteria. A 16S rRNA gene sequence-based phylogenetic analysis indicated that five of the total 27 isolates belonged to three novel species. I undertook polyphasic taxonomic analyses of four isolates and described *Olsenella umbonata* and *Veillonella magna*, a novel lactic acid-producing and -fermenting species, respectively, with now validly published name. These species were phenotypically characterised as being well adapted and, as other olsenellae and veillonellae, presumably indigenous to the gastrointestinal tract of pigs and other homeothermic vertebrates.

Zusammenfassung

Kraatz, M. (2011). Isolierung von Milchsäurebezogenen Bakterien aus dem porcinen mukosalen proximalen Gastrointestinaltrakt, einschließlich *Olsenella umbonata* sp. nov. und *Veillonella magna* sp. nov.

Ich beschloss, meine Doktorarbeit dem Thema der Isolierung von Laktobazillen und anderen Milchsäurebezogenen Bakterien (MSBB) von den Schleimhäuten der Mägen und Leerdärme gesunder Hausschweine zu widmen, da die Interaktionen dieser indigenen Mikroorganismen und Mikrohabitate äußerst relevant für die Ernährung und die Gesundheit des Wirtstieres sind.

In einem ersten Ansatz verwendete ich *Lactobacillus*-hydrogen peroxide (LHP), ein neu entwickeltes Kulturmedium zur direkten spezifischen Isolierung und qualitativen Analyse der Wasserstoffperoxid-Akkumulation von Laktobazillen in einem Schritt. In Kombination mit einem Katalase-Benzidindihydrochlorid-Screeningtest stellt LHP einen einfachen Ansatz zur Auffindung Mangankatalase-positiver Isolate potenziell neuer *Lactobacillus* Arten dar. Das neue Medium wurde als hochspezifisch und begrenzt sensitiv für Laktobazillen validiert. Die Anwendung von LHP ermöglichte die bis dato nicht berichtete Feststellung einer Häm-unabhängigen Mangankatalase im Typstamm von *Lactobacillus plantarum* subsp. *plantarum*. Ein Kolonieisolat mit einer aktiven Katalase wurde jedoch nicht aufgefunden, und so ergab dieser erste Ansatz keinen viel versprechend neuen *Lactobacillus* Stamm.

In einem zweiten Ansatz verwendete ich kommerzielles Mucin aus dem Schweinemagen als Basalkomponente zweier neu kreierter Kulturmedien für die spezifische Isolierung Schleimhautassoziierter MSBB. Die isolierte Gemeinschaft umfasste alle drei Gruppen der MSBB der *Firmicutes* und *Actinobacteria*, nämlich die Milchsäure-bildenden Bakterien *sensu stricto* und *sensu lato* sowie die metabolisch angeschlossenen Milchsäure-fermentierenden Bakterien. Eine phylogenetische Analyse auf Basis von 16S rRNA Gensequenzen zeigte, dass fünf der insgesamt 27 Isolate zu drei neuen Arten gehörten. Ich nahm polyphasische taxonomische Analysen von vier Isolaten vor und beschrieb *Olsenella umbonata* und *Veillonella magna*, eine neue Milchsäure-bildende beziehungsweise -fermentierende Art mit nun valide publiziertem Namen. Diese Arten wurden phänotypisch charakterisiert als gut adaptiert an, und wie andere Olsenellen und Veillonellen, vermutlich indigen im Magen-Darm-Trakt von Schweinen und anderen gleichwarmen Wirbeltieren.

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Statement of originality / Selbständigkeitserklärung

I hereby declare that the present thesis is my original work. I affirm that I have made use only of the indicated sources and supports. The contributions of co-authors to two chapters of this work are explained in Chapter I.

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe. Die Beiträge von Koautoren zu zwei Kapiteln dieser Arbeit sind in Kapitel I erläutert.

Mareike Kraatz Berlin, 21.1.2011