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

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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.

Keywords gastrointestinal tract; indigenous microbiota; isolation; lactic acid; mucosa-associated bacteria; swine

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 [1]. 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 [2-5]. 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 [6-8]. 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 [9].

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" [2, 8, 10, 11], presenting an entity in evolution [1, 12]. 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 [1, 13-15], whereby only 30-40 species of the "true" or "core" microbiota comprise up to 99 % of the total community and provide major metabolic activities [7, 16]. The gut microbiota can be collectively thought of as the host's largest, metabolically active entity, a veritable "microbial organ" [1]. More than other organs of the host body, this microbial organ is rapidly renewable [17], 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 [9, 18]. 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 [4].

1.3 Ecological status: autochthonous or indigenous bacteria

The GI tract is an open ecosystem in contact with the outer environment [2, 19, 20]. 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 [21] are termed "autochthonous" or "indigenous" [2, 22], meaning "found in a place where they were formed" [23] or "native to a particular habitat" [4]. These terms are widely used as synonyms [2], whereby "indigenous" is more correct [4]. 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 [17, 24, 25]. It is tempting to hypothesize that the acid-tolerant "milk

souring" lactic acid-producing bacteria (LAB) [26] 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 [2, 27]. Their timely permanent or resident presence is often due to an intimate association with the gut mucosal microhabitats [2, 28]. 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 [2, 14]. Consequently, indigenous bacteria are of inherent metabolic significance, i.e. they necessarily accomplish a demonstrable ecological function in the GI tract [27].

1.4 Pig-gut microbiota symbiosis

Under natural conditions, the pig's life is obligately dependent on symbiosis of its indigenous gut microbiota [8, 14, 27]. 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 [9, 14, 29, 30]. As, in the GI tract, the ecological relationship of any individual microbial organism is intricate and condition-dependent [30], life in coexistence with gut microbiota is a delicate balance between health and disease [6, 31].

The relationship between the pig host and its indigenous GI microbiota is generally considered to be mutually beneficial or mutualistic [9, 32]. The indigenous gut microbiota are essential mutualists for pig health and survival, at least in the absence of a sterile environment [6, 30]. 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) [20]. Taken individually, the vast majority of bacterial species in the GI tract would be considered as commensals [27]. The term "commensalism" literally means life "at table together" [16]. A commensal species benefits by partaking nutrients and exploiting a niche, without negatively or positively influencing its host [1, 4].

The symbiotic pig-gut microbiota relationship may be described as "covert parasitism" [27], 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 [3]. However, once the homeostatic mechanisms are disrupted under certain circumstances, the potential pathogens may easily overgrow [28] and the indigenous microbiota be a source of endogenous or opportunistic infection and disease [14, 18, 33].

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 [6, 7, 34]. Germfree animals show significant local (GI tract) and systemic (body metabolism) differences compared with their naturally raised counterparts [35, 36]. The GI phenotypes that are characteristic of conventionally colonised hosts in contrast to germfree hosts are collectively known as "microflora associated characteristics" [16, 31]. The ecological functions that are provided by the indigenous microbiota can be put into two groups: nutritional and protective functions [6].

Growth promotion due to enhanced nutrient exploitation and energy harvest is the most prominent benefit from nutritional functions [37-39]. Germfree animals require a higher dietary caloric intake than conventional animals [35]. The "microbial balance index" [40] 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 [41] relies on the "niche exclusion principle" of Hardin [42]. 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 [6, 14, 28, 43]. The resistance of climax microbial communities to structural change following perturbation is based on the functional redundancy of phylogenetically diverse species populations [30].

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 [44]. 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 [45]. 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 [26]. Whereas this phenotypic definition is generally valid even today [44], the introduction of modern molecular genetic methods into bacterial taxonomy revealed that LAB do not form a "great natural group" [26], but are widely allocated on two disparate phylogenetic lineages of the Gram-stain-positive bacteria, namely the phyla *Firmicutes* and *Actinobacteria* [46]. The traditional definition of LAB is phylogenetically restricted in that it generally excludes the *Actinobacteria* [23, 47]. However, as pointed out by Axelsson in 2004 [44], 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 [4, 48]. 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 [49]. 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 [50]. 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 [2, 27, 37].

The GI tract is equivalent to a chemostat with steady state conditions [31, 43]. 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 [14, 30], complex food webs of lactic acid-related bacteria (LARB) present an intrinsic factor promoting gut ecosystem structural and functional stability [51]. Food chains of LARB comprise a main example of "niche construction" through synergistic nutritional interactions (syntrophism or interspecies cross-feeding) [14, 15]. 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 [4, 7, 49].

The Gram-stain-positive LAB can be grouped into the "LAB *sensu stricto*" and the "LAB *sensu lato*" [48]. Both groups are collectively termed "lactic microbiota" [23] and, at the time of writing, comprise around 50 genera [48].

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 % [46, 48]. This low-G+C-content group of LAB comprises the historic "milk-souring organisms" of Orla-Jensen [26], with the core genera *Lactobacillus*, *Leuconostoc*, *Weissella*, *Pediococcus*, *Streptococcus* as well as *Lactococcus* and *Enterococcus* and, in the pig GI tract, also *Gemella* [13, 19, 44]. 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 [52]. 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 [26, 45]. 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 [44, 53].

Due to the "special foregut association" [54], i.e. formation of true biofilms on the epithelial surfaces of the oesophagus and the pars oesophagea of the stomach [55], many lactobacilli and other LAB *sensu stricto* belong to the indigenous bacteria in the pig GI tract [15, 23, 33]. Lactobacilli are generally regarded as safe commensals with no distinct pathogenic potential for humans or animals [19, 38, 45, 53]. 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 [44]. The fermentative properties of gut bacteria are in the focus when elucidating their ecological role for pig health [56]. The LAB are regarded the most beneficial part of the indigenous gut microbiota [57]. The presence of lactobacilli and other LAB in the GI tract is generally considered to be advantageous to the pig host [58], whereby an increased lactobacilli:enterococci ratio has been suggested to serve as an index of the health status [59].

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) [60] 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* [48]. Bifidobacteria such as "*Bacterium bifidum*" [26] are historically considered to belong to the LAB [44]. 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 [46, 53, 55]. 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* [61]. Both genera contain species that were reclassified from *Eubacterium*, *Lactobacillus* or *Streptococcus* [62, 63]. 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) [64, 65].

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 [51]. 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 [1, 7, 9]. 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 [66-70], 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 [71, 72]. 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 [1, 15, 30, 43].

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 [72]. According to Chassard and co-workers [51], 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 [73].

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 [2]. 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 [17, 20, 37, 49, 55, 58, 67, 74-76]). 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 [49, 73, 77-79]. Together, LAB and the functionally specialised lactic acid-fermenting bacteria constitute the pioneering group of LARB in the "microbial ecosystem" [80] 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 [2, 80]. 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 [77, 78, 81]. More recently, the introduction of molecular genetic methods into gut bacteriology has confirmed the earlier findings [6, 13]. 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 [23, 37, 74-76]. LAB are among the predominant mucosa-associated bacteria in the overall GI tract of adult pigs [7, 59]. In concomitance with LAB, lactic acid-fermenting bacteria of the genus *Veillonella* are found regularly as indigenous inhabitants of all gut sections (reviewed by [82]).

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 [17]. 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 [2, 7]. Unlike the bulk of gut microbes, LAB and especially lactobacilli are acid-tolerant

[28, 44, 55] and generally able to adhere to and colonise the mucosal surfaces [67]. 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 [6, 19, 37, 83-86].

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 [8, 12]. Pigs and humans are omnivores possessing a continuous (straight-tube), simple GI system without a functional caecum [69, 87, 88]. 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 [2, 37, 69]. 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 [14, 69]. 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 [69, 89].

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 [90] and encompass two of the three main digestive sections, with a total volume capacity of about each 30 % [69, 87]. The pig stomach is of the one-cavity and compound type [69, 88, 90]. 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 %) [91-93]. The stomach of pigs is the site for food storage and, after the mouth, the second stage of mechanical and initial endogenous enzymatic digestion [8, 87]. The small intestine of pigs is comprised of the duodenum, the jejunum and the ileum from cranial to caudal end [90]. In the fully grown pig, it generally approximates 18 meters with almost 90 % as jejunum [87, 92]. The jejunum is the site where the majority of endogenous digestion ensues and the overwhelming proportion of nutrient absorption [28, 87]. Remarkably, despite their physiological importance, only little is known about the bacterial colonisation of the pig stomach and jejunum [66, 94].

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 [93, 95, 96]. 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 [14, 28]. 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 [96, 97]. 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 [14, 97]. 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 [87, 90, 92, 95, 96, 98]. The mucus gel bilayer serves as a protective lubricant and is part of the unspecific, innate host defence system [14, 93]. 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 [14, 93, 95].

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 [28, 67]. In pigs, only the distal ileum and the large intestine exhibit prolonged stasis allowing the luminal digesta to be colonised [2]. 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 [4, 7, 14, 73, 99, 100]. Indigenous bacteria of these “lotic” habitats are necessarily directly adhered to or indirectly associated with the mucosal surfaces [7, 28, 55, 76]. 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 [9, 57]. 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 [9, 14, 28, 43]. 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 [14, 28, 101-103].

In pigs, the surfaces of the stomach and small intestine are densely populated with indigenous mucosa-associated bacteria [37, 59, 79], including lactobacilli and other LAB *sensu stricto* [22, 54, 75, 76, 104] that markedly interact with the structure of the GI mucosae [57]. 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 [6, 45, 55].

3.5 Coaggregation, microcolonies and biofilms

Specific lectin-carbohydrate interactions are usually the basis for interbacterial adherence (coaggregation) [105]. Coaggregation facilitates the formation of multicellular microcolonies on the GI epithelial surfaces [14]. Microcolonies are the predominant colonisation form in the human gut [38]. 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 [14, 43]. Microcolonies are often embedded in a matrix of bacterial extracellular polymeric substances, usually exopolysaccharides [14, 38, 39, 54]. 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 [14, 105], rather than on the secretory columnar epithelia of the glandular stomach and small intestine [106]. 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 [7, 8, 31, 38, 54, 55].

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 [22, 107]. 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 [19, 23, 67, 81]. Some intestinal lactobacilli exhibit cell surface polysaccharides, mostly exopolysaccharides, which resemble host mucins in the composition of the oligosaccharide side chains [38, 39]. 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* [38, 39, 108]. 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 [33, 109]. 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") [6, 14, 30, 45, 57, 73].

4. Digestive life of bacteria on exogenous nutrients

Lactobacilli and other gut LARB are chemo-organotrophic and typically auxotroph organisms with fastidious nutritional requirements [44, 46]. They are generally associated with habitats that are rich in easily assimilable substrates, such as amino acids, simple carbohydrates and vitamins [23, 44]. 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 [68]. 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 [54, 87].

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 [69, 87]. 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 [88], 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 [69, 98].

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 [69]. Pancreatic enzymes include endo- and exopeptidases (trypsin, chymotrypsin, elastase, carboxypeptidases A und B), α-amylase, lipases (lipase, colipase, phospholipase A_2, 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 [69, 98]. 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 [88]. 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 [88]. 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 [87]. 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 [98].

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 [85]. Members of the mucosal gut microbiota affect intestinal epithelial physiology, including the activities of brush-border enzymes [32]. They modify the epithelial expression of several host genes involved in the processing, absorption and metabolism of carbohydrates, lipids and micronutrients [8, 14]. *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 [16, 39, 99, 110, 111]. Microbial metabolism in the pig mucosal proximal GI tract is an important factor on host systemic metabolism and energy storage [8, 110, 112].

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 [55, 77, 87, 113]. Following digestion, the proximal GI tract is purified from residual nutrients by interdigestive propulsive activities [98]. Prolonged interdigestive periods (fasting) cause a marked reduction but not complete decolonisation of LAB and other indigenous bacteria [77, 84, 114]. Rapid reconstitution of community patterns is assured by the mucosa-associated populations [103] that rely on mucin glycoproteins as endogenous continuously available nutrients [16, 19, 51, 108]. Mucins are the main macromolecular structural constituents of the aqueous mucus gel bilayers [14]. A great number of indigenous bacteria have coevolved the capability of utilising mucins as alternative sources of carbon, nitrogen and energy [14, 51, 115]. 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 [16, 18, 28]. 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 [14, 89, 116].

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 [1, 30, 105, 117]. Providing continuously present metabolic sites (niches) for diverse bacterial communities, mucins accomplish an ecologically outstanding stabiliser function in the interdigestive proximal GI tract [1, 35]. More diverse bacterial communities that occupy all niches in an ecosystem generally exhibit greater structural and functional robustness [28, 30, 43]. 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 [1, 17].

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 [14]. 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 [14, 43]. Thus the mucosal niches of the pig stomach and small intestine are determined by a range of adverse mechanical, biological and physicochemical environmental factors [14]. Autochthony or indigeneity is based on the presence of genes for factors that allow bacteria to overcome adverse environmental conditions [54]. 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 [118, 119].

Adverse mechanical factors include removal forces due to digestive and interdigestive gut motor functions and propulsive activities (peristalsis) [2, 4, 43, 98], villous motility of the small intestinal mucosa [101] and shedding and renewal (turn over) of the epithelial cell surfaces [87, 93]. 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 [8, 95] as well as different immune responses of the gut-associated lymphoid tissue [14, 120]. Important physicochemical environmental factors are the pH as well as oxygen and redox potential [14].

6.1 Oxygen as an omnipresent physicochemical factor

Oxygen molecules generally pass from the blood through the gut epithelia into the mucosal microhabitats [2]. The stomach and small intestine of pigs are highly vascularised organs, and, therefore, close to the mucosa the oxygen contents are relatively high [19]. In the stomach, the secretory mucosa of the oxyntic gland region is rich in alveolate webs of blood capillaries [95] in order to sustain the energy-consuming secretion of HCl [87]. Besides, small amounts of oxygen in air are regularly swallowed into the stomach by the intake of food or water [14]. 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 [14]. The absorptive mucosa of the jejunum exhibits a very tight microvascular epithelial network [25, 87, 95, 121, 122]. The oxygen content at the luminal surface of the small intestinal mucosa corresponds to about 22 % of the oxygen content of air [14].

Because of its strong oxidising ability, the presence of oxygen exerts a severe effect on the redox potential in the environment [14]. Indigenous lactobacilli and other bacteria encounter redox stress conditions associated with the oxygen gradients that are steep at the mucosal surfaces [39]. The oxygen content generally declines towards the gut lumen due to reduction by oxygen-tolerant microorganisms [14]. 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 [20, 44, 123].

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?" [6, 30, 31]. They remain the "gold standard" for isolating strains of novel species for representative phenotypic, genetic and molecular characterisation [9, 100, 124]. The predominant part of the pig GI tract bacteriobiota has not yet been recovered in pure culture [6, 8, 13].

7.1 Specific isolation of haem-independent catalase-positive lactobacilli

The pig proximal GI tract harbours a major proportion of presumably unknown *Lactobacillus* species [125]. Detection of isolates of novel species is likely to succeed best using alternative specific culture media and specifically adjusted conditions [53, 126]. 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 [127], however, lactobacilli usually do not possess catalases and are poorly equipped with other antioxidative enzymes for the degradation of accumulating hydrogen peroxide [53, 128]. 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 [129] 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* [66]. Mucosa-associated bacteria often take more time to grow on conventional media and, therefore, require more advanced, innovative cultural approaches [85]. More advanced culture techniques incorporate various nutrients and abiotic conditions that closely mimic the environment from which the bacteria were isolated [30]. 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* [61, 82].

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