CHARACTERIZATION OF THE DEVELOPMENT OF THE TONSILLAR MICROBIOME IN PIGS

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ABSTRACT

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Pig tonsils are identified as a potential reservoir for many bacterial and viral pathogens that can survive asymptomatically in this location and may have a high potential of being zoonotic. It has been suggested that the microbiome plays a significant/ substantial role in host colonization by pathogenic microorganisms and also exerts regulatory roles in the resistance to infection. Despite the important role that the tonsillar microbiome could play in the colonization and persistence of pathogens in the host, there are no in-depth studies characterizing the development of tonsillar microbiome in pigs or how this microbiome is structured over time. Surprisingly a similar study is also absent in humans. There was a clear need to investigate the development of the tonsillar microbiome in pigs to lay the basis for future studies focused on more complex subjects such as the relationship between the normal tonsillar microbiome and pathogens in the tonsils. Understanding the development of the pig tonsillar microbiome over time and the role of the tonsillar microbiome in the acquisition and persistence of a pathogenic microorganism will lay the basis for the design of novel intervention strategies to control the presence of the pathogen and reduce the risk of transmission to other animals or humans. Moreover, these studies are expected to provide an animal model to test hypotheses generated by microbiome data that cannot be tested in humans. The goal of this study was to characterize tonsillar microbiome development in pigs, and how this microbiome is structured and how the structure changes through different times in the life of pigs. The

chapters in this thesis will present pertinent data related with the composition of the pig tonsillar microbiome and how it alters through the life of pigs, possible maternal sources for some of the identified members of this microbiome, as well as the microbiome structure and progressive change through their life. Furthermore, the results will show that challenges associated with management procedures typically present in swine farms generate prominent changes in the microbiome composition and abundance of diverse bacterial families. Finally, the study will show the microscopic structure of the tonsillar epithelium and crypts and the presence of diverse bacterial communities on the surface of pig tonsils throughout different time points of their life. The final chapter will also describe the morphological changes of the tonsillar surface in pigs that are seen and are associated with changes in the microbial communities observed through different time points in their life. Taken together, the results presented here demonstrate that there is a temporal succession in the development of the pig tonsillar microbiome through the life of pigs.

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KEY ABBREVIATIONS

rRNA	ribosomal Ribonucleic acid
OTUs	Operational Taxonomic Units
SIMPER	Similarity-Percentage
FISH	Fluorescent In Situ Hibridization
CLSM	Confocal Laser Scanning Microscopy
SEM	Scanning Electron Microscopy

CHAPTER I. INTRODUCTION AND LITERATURE REVIEW

Recently humans have become more aware of the role played by bacterial communities in human and animal health. These bacterial communities can reduce acquisition of pathogens by competitive exclusion, improve many physiological functions and play a complex role in shaping/regulating tissue homeostasis and health. In the last few years since the advent of advanced sequencing technologies and increased affordability, there has been a bloom in studies identifying the presence of a normal bacterial population in different hosts and host tissues. Sequencing has enabled identification of complex relationships between diverse bacterial populations in host tissues, including the presence of pathogenic microbes and the relationship of the normal microbiota to other microbes and disease states. For example, a recent study showed how the presence of bacterial members of order *Clostridiales* enhanced the resistance of mice to experimental infections with enteric pathogens such as *Salmonella enterica Typhimurium* and *Citrobacter rodentium* [1].

It is broadly acknowledged that tonsils act as a first line of defense, continually surveilling for pathogens entering the host through the nasal or oral routes. Further, tonsils function as a site of colonization and primary replication for bacterial and viral pathogens that can be host-specific or zoonotic organisms transmitted from animals to humans [2]. Currently, there are no studies identifying the interaction of the normal microbial population in human tonsils with pathogenic microorganisms and, more importantly, no worthy animal models to study and test hypotheses about relationships between normal tonsillar microbiota and pathogenic bacteria in humans have been described.

In this review, we will briefly discuss the use and role of animal models to study bacterial respiratory diseases in humans, particularly the use of pigs to study diseases in humans. The review will further discuss tonsils, including their structure and their role and relevance as a first barrier to prevent the colonization of the host by pathogenic bacteria. Additionally, the review will elucidate some particularities of tonsillar development in human and pigs. Finally, the review will summarize the current base of knowledge of microbial populations in human and pig tonsils.

ANIMAL MODELS AND THE STUDY OF HUMAN RESPIRATORY DISEASES.

Animals have been used as models to study human diseases for many years, and have helped researchers fathom the mechanisms of pathogenesis of some diseases as well as to develop therapies to cure those conditions in humans [3, 4]. Many of the "traditional" models are selected based on simplicity to manage and celerity to conduct studies, sometimes leaving aside the intricacies of certain physiological aspects [4]. One of the most common animal models used is the humanized mouse model; however this model frequently does not mimic the human conditions [3]. This becomes more relevant when working on microorganism-associated disease, due to the fact that mice and other laboratory species are usually not infected by microorganisms that infect humans [5]. Other cheap or easier to handle models broadly used in comparison/translational studies are the rodent models such as rat and guinea pigs. Although these rodents are readily available, their lack of differentiated tonsillar tissue [6, 7] makes them a very poor model for tonsillar microbiome research.

There is not an animal model that meets all the specific requirements for a translational study [8]. The selection of a good animal model depends on the special conditions required for the study. The pig has been used as a relevant model for a wide range of human diseases, such as atherosclerosis, diabetes, and skin disease, because of the similarities it shares with humans, including anatomy, physiology, organ development and disease progression [3, 4, 9].

Growing interest in the use of pigs as a translational model have been the object of international interest represented in symposiums to discuss their prospective uses, advantages and disadvantages [8, 10, 11]. Examples of the use of pigs as a translational model to provide new understanding of the pathogenesis of relevant disease affecting humans are multiple. Pigs have been genetically modified to generate models of cystic fibrosis (an inherited and fatal disease of Caucasians), providing better approaches to understand the disease mechanism, especially based on the organs involved in the pathology, which is not provided by the existing mouse models [12-14]. Pigs have been used as model to study organic responses to *Pseudomonas aeruginosa* in sepsis [15] and in ventilator-associated pneumonia [16] as well as in lung infections [17]. Pigs have been used to study the pathogenesis of influenza and infection with secondary bacterial pathogens such as *Bordetella bronchiseptica* [18] or *Staphylococcus aureus* [19], as well as a model to study the pathogenesis of respiratory infection by *Bordetella pertussis* [20] and *Bordetella parapertussis* [21].

Humans and pigs share significant similarities in anatomical features of the upper respiratory tract, such as the arrangement of the pharyngeal “Waldeyer´s ring” lymphoid tissue. With the exception of primates, the pig resembles the human upper respiratory

anatomy most closely [2]. Further, pigs have a distinct advantage over rodents due to the high similarity of immune parameters with humans (higher than 80%, compared with less than 10% of similarity that has been reported for mice) [9].

Pigs and humans share common pathogens that are able to induce disease or be transmitted between the two species. Examples of such pathogens include *Streptococcus suis*, which is an important respiratory and neurologic pathogen of pigs that is now identified as a cause of meningitis in humans worldwide and an important emerging disease of humans [22-39], and influenza, a viral pathogen shared with humans [40-49]. The fact that many respiratory pathogens have similar pathogenic mechanisms in humans and pigs has led to increased use of pigs as a model to study human respiratory pathogens [9].

Tonsils have been reported as a primary portal of entrance and colonization for some of these pathogens, from where they are routinely isolated [50]. Studies of bacterial interactions with normal tonsils in humans are complicated due to the reported difficulties in obtaining normal human tonsillar tissue [51] and the inability to study the effects of acquisition of pathogens in humans. Human tonsil explants have been used to study the interactions of human tissue with diverse infectious agents [52]. However, this model lacks certain aspects present in normal tissue interactions, such as the presence of an intact immune system. The potential contamination inherent to the non-sterile nature of these explants is another serious problem [52]. Because of the high similarities between pig and human tonsils, described below, it is reasonable to propose the use of pigs as a good exploratory translational model to study the tonsillar microbiome and to test hypotheses about the interaction between tonsils and the microbiome.

ANATOMY AND HISTOLOGY OF TONSILS

The pharyngeal region of some species is characterized by the presence of lymphoid cell aggregations located in the wall of its lamina propria. These aggregations are called tonsils [53], and include the lingual tonsils, palatine tonsils, paraepiglottic tonsils, pharyngeal tonsils, tubal tonsils and the tonsils of soft palate. From the aforementioned, the single nasopharyngeal tonsils or adenoid, lingual tonsil, paired palatine and tubal tonsils, form the vast majority of the subepithelial secondary lymphoid organs located in pharynx known as Waldeyer´s ring [53], which have been suggested to play a role in local B cell dissemination [54]. The number of tonsils present in each species and the features of each one can vary widely (Table 1.1.)

First, it is important to mention the confusion related to the existence of palatine tonsils in pigs, which have been clarified recently by Casteleyn et al [55, 56] to actually be tonsils of the soft palate and not palatine tonsils. Previous to Casteleyn´s detailed report on the structure of tonsils in many species of mammals, some reference text books [57] as well as research papers [50, 58-63] regularly referred to the use of palatine tonsils in pigs for their research work. Because of that, most of the references previous to Casteleyn´s report in 2011 can generate a confusion. We will use the term tonsils of the soft palate in this review.

Tonsils as aggregations of mucosa-associated lymphoid tissue (MALT) are formed by areas of B-cell collections (lymphoid follicles) surrounded by T-cells (interfollicular regions) [53]. Approximately 90% of tonsils are comprised of lymphoid tissue [64], clustered as

Table 1.1. Characteristics from the main tonsils identified in different species

Tonsil	Lingual	Palatine	Paraepiglotic
Sheep	Small aggregations of lymphoid cells, located in the oropharynx, at the lateral sides of the root of the tongue. Non visible macroscopically. Covered by keratinized stratified squamous epithelium.	Ovoid structures bilaterally located in oropharynx, between palatoglosal and palatopharyngeal arches. Possess 1 -3 crypt orifices.Covered by a nonkeratinized stratified squamous epithelium.	Nodular mucosal elevations located bilaterally in the laryngopharynx, lateral to the base of epiglottis. Covered by a slightly ketarinized stratified squamous epithelium.
Goat	Small aggregations of lymphoid cells, located between lingual muscle and the salivary glands in the region of the vallate pappillae. Not visible macroscopically. Covered by nonkeratinized stratified squamous epithelium.	Ovoid structures bilaterally located in oropharynx, between palatoglosal and palatopharyngeal arches. Few crypt openings. Are larger than homologous in sheep. Covered by a nonkeratinized stratified squamous epithelium.	Hardly visible macroscopically in few animals. Evident as small mucosal elevations located bilaterally in the laryngopharynx, lateral to the base of epiglottis. Covered by a slightly ketarinized stratified squamous epithelium.
Ox	Well developed and visible macroscopically, located rostrolaterally at the root of the tongue. Possess multiple rows of crypts. Covered by a ketarinized stratified squamous epithelium .	Large ovoid and bilobated structures, bilaterally located at the oropharyngeal surface, between palatoglosal and palatopharyngeal arches. Possess multiple crypts. Covered by a stratified squamous epithelium.	Absent
Pig	Prominent lymphoid clusters located at the base of the tongue, caudal to the foliate papillae. Possess deep crypts. Covered by slightly keratinized stratified epithelium.	Absent	Round to oval plaque located bilaterally cranioventral to the base of epiglottis. Possess few crypts. Covered by squamous stratified epithelium.
Horse	Macroscopically visible as multiple fossules (crypt entrances) located at the dorsolateral sides of the root of the tongue. Covered by a keratinized stratified squamous epithelium.	Elongated flat strucutre located bilaterally on the floor of the oropharynx, caudolateral to the tongue. Multiple crypt entrances are visible. Covered by a slightly keratinized stratified squamous epithelium.	Absent
Dog	Disseminated aggregations of lymphoid cells located at the base of the tongue. Covered by keratinized stratified squamous epithelium.	Located bilaterally in the lateral sides of the oropharynx, between the palatoglossal and palatopharyngeal arches. Covered by a thin stratified squamous epithelium. Possess one rypt.	Absent
Cat	Poorly developed aggregations of lymphoid cells that cannot be observed macroscopically. Located in the mucosa of the root of the tongue. Covered by a keratinized squamous epithelium.	Oval shaped aggragations of lymphoid tissue. Possess a central entrance to crypt. Covered by keratinized squamous epithelium.	Vary between individuals, but well developed tonsils can be identify as mucosal elevations located bilaterally cranioventral to the base of epiglottis. Covered by a thin stratified squamous epithelium.
Rabbits	Absent	Bilateral oval protrusions, located in the dorsolateral wall of the oropharynx. Possess one crypt entrance. Covered by a keratinized squamous epithelium.	Absent
Human*	Located on the posterior third of the tongue. Covered by partially keratinized squamous stratified epithelium. Possess a single crypt.	Bilaterally located in the oropharynx. Covered by nonkeratinized squamous stratified epithelium. Possess branching crypts.	Absent

All the data contained in this table, except * was extracted from [56]. * data was extracted from [53]

Table 1.1. (cont´d).

Tonsil	Pharyngeal	Tubal	Soft palate
Sheep	Prominent structure located dorsally in nasopharynx on the caudal part of pharyngeal septum. Covered by a pseudostratified columnar ciliated epithelium. Possess crypts.	Scattered nodules (not easily visible macroscopically without tissue pretreatment), bilaterally located in the nasopharyngeal wall, around and caudoventral to the opening of auditory tube. Covered by pseudostratified columnar ciliated epithelium.	Scattered nodules (not easy visible macroscopically without tissue pretreatment), located at the dorsal surface (nasopharyngeal side) of the soft palate. Covered by connective tissue and respiratory epithelium rostrally, and by stratified squamous epithelium in middle part.
Goat	Prominent structure located dorsally in nasopharynx on the caudal part of pharyngeal septum. Covered by a pseudostratified columnar ciliated epithelium. Possess crypts.	Not visible macroscopically, bilaterally located in the nasopharyngeal wall, around and caudoventral to the opening of auditory tube. Covered by pseudostratified columnar ciliated epithelium.	Less prominent than the homologue in sheep (not easily visible macroscopically without tissue pretreatment), located at the dorsal surface (nasopharyngeal side) of the soft palate. Covered by pseudostratified columnar ciliated epithelium rostrally, and by stratified squamous epithelium in caudal part.
Ox	Prominent structure, located dorsally in nasopharynx on the caudal part of pharyngeal septum. Sometimes epitheliuml infolds form crypts. Covered by a pseudostratified columnar ciliated epithelium.	Visible as a cluster of irregular mucosal elevations, located bilaterally caudoventral to the opening of the auditory tube. Covered by pseudostratified columnar ciliated epithelium.	Diffuse and scattered tissue bilaterally located in the nasopharyngeal side of the soft palate. Covered by pseudostratified columnar ciliated epithelium.
Pig	Macroscopically visible as a prominence of the caudal end of the pharyngeal septum. Possess crypts. Covered by a pseudostratified columnar ciliated epithelium.	Visible macroscopically as a irregularity of the nasopharyngeal mucosal surface, located bilaterally caudoventral to the orifice of auditory tube. Possess a central crypt. Covered by a pseudostratified columnar ciliated epithelium.	Well developed oval bilateral plaque. Located in the ventral (oropharyngeal) side of the soft palate. Possess multiple crypts. Covered by a slightly keratinized stratified squamous epithelium.
Horse	Macroscopically hardly visible and not well delineated. Located diffussely in the nasopharyngeal wall. Covered by pseudostratified columnar ciliated epithelium.	Diffuse lymphoid tissue located near the opening of the auditory tube but continuous with the pharygeal tonsil. Impossible to delineate exact limits with pharyngeal tonsil. Covered by a pseudostratified columnar ciliated epithelium.	Prominent slightly raised oval area, located centrally at the ventral side of the soft palate (oropharyngeal side). Possess multiple crypts.
Dog	No prominent flattened aggragations of lymphoid tissue located in the nasopharynx, dorsal to the openings of the auditory tubes. Doesn´t have crypt entrances (fossules). Covered by pseudostratified columnar ciliated epithelium.	Absent	Absent
Cat	No prominent flattened aggregations of lymphoid tissue located in the nasopharynx, dorsal to the openings of the auditory tubes. Doesn´t have crypt entrances (fossules). Covered by pseudostratified columnar ciliated epithelium.	Absent	Absent
Rabbits	Absent	Absent	Absent
Human*	Nasopharyngeal tonsil or adenoid, is depicted as an ovoid shape structure located on the roof of the nasopharynx. Covered by pseudostratified columnar ciliated epithelium.	Bilaterally located at the pharyngeal opening of the Eustachian tubes. Covered by pseudostratified columnar ciliated epithelium.	Absent

All the data contained in this table, except * was extracted from [56]. * data was extracted from [53]

lymphoid follicles where B cells mature and differentiate and T cells are activated, while the interfollicular regions are the location of specialized venules (High Endothelial Venules: HEV) which play a role in the migration of T and B cell from blood to tonsils [65].

The lymphoid follicles are spread uniformly in the subepithelial mucosal layer [64] and have been classified as primary lymphoid follicles and secondary lymphoid follicles. Primary follicles, or resting follicles, are not involved in response to antigens [66], while secondary follicles are busily involved in antigen-driven processes [67] Within the secondary follicles, immune activated B cells proliferate centrally. The B-cells also form zones with diverse degrees of differentiation and stratification known as dark, light and mantle zones [68]. The anatomical location makes the palatine tonsils easily accessible thereby making them one of the most widely studied lymphoid organs to understand interactions of immunological cells and antigens [65]. The remainder of this review will primarily focus on these particular tonsils for humans and the tonsils of the soft palate for pigs and their resident microbiome.

Palatine tonsils play an important role in immune defense, preventing foreign antigens from entering the organism via the respiratory and gastrointestinal pathways [69, 70], suggesting an active role as one of the first components of the immune system to be exposed to antigenic stimulation after birth [71]. The tonsil surface possesses thin channels known as crypts, which facilitate the interaction between foreign antigens and the immune system [72].Their numbers vary between species and type of tonsils. For palatine tonsils, in particular, cats do not have crypts [73], rabbits only have one [7], ovine have 1 – 3 [74], and humans possess 10 – 30 [75], while the tonsils of soft

palate in pigs have between 160 -190 [76]. These crypts are present at birth, and the length and size of tonsillar crypts continues to grow after birth [77]. The crypt shape can vary between species, however tonsils of human and pigs have characteristic tubular crypts [76, 78]. Tonsils act as a first interacting organ with antigens entering by the oral route [79], where the epithelial layer lining the tonsillar surface and crypts plays a critical role in sensing antigens [80]. Because tonsillar lymphoid follicles lack afferent lymphatic vessels, the intraepithelial passage-ways present in the reticular epithelium allow the flow of intercellular fluid and lymphoid cells, which will finally reach lymphatic vessels and follicles [81].

Ultrastructural studies of the epithelial surface in palatine tonsils have shown that there are two distinguishable types of epithelium lining the tonsil surface, reticular and non-reticular [80, 82]. The non-reticular epithelium is composed of non-keratinized stratified squamous cells that cover the tonsillar surface and entrance of the crypts. The non-reticular epithelial cells have characteristic microscopic folds on the surface, whose shape varies and is more noticeable through the crypt extension, especially in the deeper parts of the crypt. A second and major component, covering most of the crypt is a reticular epithelium invaded by lymphoid cells [76, 82]. In the junction of the borders of both epithelia, the borders of the reticular epithelial are depressed and might provide a surface for bacteria to adhere to [82].

The epithelium lining the tonsil surface is formed by polygonal cells whose apical surface is covered by patterns of characteristic microscopic folds (microridges) [83], or identified as microvilli or microplications on the surface of epithelium [76], which may assist bacterial attachment [83], since it has been demonstrated that the bacteria,

Streptococcus pyogenes, use the crest of these microridges for initial attachment, a first step in the colonization process [84]. Another characteristic cell, the fungiform cell [82] has been described and appears to be the same cell identified by other authors as the specialized surface cell (M cell), characterized by the presence of microvilli which suggest a critical role in the antigen uptake [85]. The M cells vary morphologically, but frequently are characterized by large and developed folds of the apical membrane [76, 80] that are tightly associated to intraepithelial lymphocytes [76]. The characteristic shape of M cells seems to provide a large contact surface, facilitating the uptake of foreign material [80]. M cells were found disseminated through the crypt epithelium especially at projections of the crypt surface and comprise up to 35% of epithelial cells [86] in the tonsillar crypts. They play a significant role in mucosal defense [80], being capable to transporting antigens across the epithelium [87]. It has been proposed that tonsillar M cells are constantly sampling antigens, such as bacteria and other microbes which are continuous tonsillar colonizers, and may assist with tolerance and defense mechanisms, either local or systemic, against these microorganisms [88]

Tonsillar crypts provide a large surface area for antigen contact [75], and the stratified squamous epithelia lining the crypts of palatine tonsils are characterized by the presence of multiple channels infiltrated by diverse cells including lymphocytes, plasma cells and mononuclear phagocytic cells [85]. Abundance of infiltrating non-epithelial cells (specially lymphocytes B and T), macrophages, dendritic langerhans cells and Natural Killer (NK) cells and intraepithelial small vessels scattered through epithelial layer [89, 90] have been reported, as well as the presence of neutrophils in the crypt lumen [90]. Goblet cells, characterized by the presence of mucous cytoplasmic

granules, have also been described in the middle and terminal regions of the crypts, often tightly associated to M cells [76]. Also, diverse leucocytes, epithelial cells, Periodic acid-Shiff (PAS) positive content (polysaccharides and mucosubstances) and free or phagocytosed bacteria have been observed in the lumen of crypts [91] and seems to be sloughed off from the area [64].

TONSIL DEVELOPMENT

Human tonsil development. The second branchial pouch, known to be the precursor of palatine tonsils in the fetus, is identifiable in the first month of gestation and by the second month the epithelium begins to evaginate to form the early tonsillar fossa and there is evidence of canalization and branching into early crypts present in the structure [92]. By the seventh month of gestation there is evidence of lymphoid infiltration into the lamina propria, and by the last trimester of gestation, the primary follicles can be identified [92]. However, newborn infants lack tonsillar lymphoid follicles, which are numerous in adults [93].

The tonsillar crypt structure in humans has been reported as beginning to appear at the third month of fetus development and to reach an approximately constant number at birth [77]. Minear et al. showed that the number of crypts seems to be constant even in adulthood, reaching a maximum of thirty eight [77]. This is in contrast to the studies done by Kassay and Sandor which reported that the age and size of the tonsil have a higher influence on the numbers of crypts observed, with the number of crypts varying from 16 to up to 25, depending on whether it is a small or a giant tonsil

[75]. However, more important than the number of crypts are the number and length of the crypt branches, which expands the surface area of the tonsils significantly [75].

Tonsillar germinal centers appear only after birth once they are stimulated by antigens and continue to proliferate during the first year of life [92, 94], reaching their highest abundance during the first 2-3 years of childhood and declining around 8 – 14 years [95], they revert with age, which involves a decrease in the proliferating pool of the B cell population [96]. It has been shown that the presence of immunoglobulin positive cells in tonsils is negatively correlated with age, meaning that number of cells positive for immunoglobulin decreases as age increases [69].

Human tonsils are characterized by the absence of sinuses and the presence of epithelial infiltrates of macrophages and lymphoid cells [97], where the luminal side of the mucosa is particularly populated by B cells and the apical side by mature plasma cells predominantly positive for IgG and in lower numbers positive to IgA, IgM and IgD [90]. This epithelial cell infiltration and the presence of pores in the basement membrane of the tonsillar crypt epithelium is a natural developmental finding in prenatal and postnatal human crypts [98]

Immunoglobulin production by cells from normal human palatine tonsils was initially investigated by Brandtzaeg et al. [99] and they demonstrated that B cells differentiate into IgG blast-cells in the germinal centers of tonsils, and that this B cell system is highly activated throughout human life. The B cell system plays a significant role in identifying foreign material and driving clonal expansion and differentiation, in order to control antigenic agents [98, 99]. It has been demonstrated that tonsils have a

role in the generation of local and disseminated antibody responses, and contribute to the development of immunological memory [100].

Pig tonsil development. The pig tonsils also arise from the pharyngeal pouches [101], as in humans. The first evidence of formation of tonsillar tissue appears in fetuses of 90 mm (approximately 70/114 days of gestation), evidence of crypts appears in the 142 mm stage (approximately 80/114 days of gestation), and defined lymphoid nodules appear in the 275 mm stage (approximately 110/114 days of gestation) [101]. Germinal centers in tonsils are absent in germ-free piglets under 4 weeks of age [102]. However, the presence of bacterial antigens is able to induce the formation of germinal centers in tonsillar tissue [103]. In tonsils, the lymphoid follicles develop as piglets age, where 1 week old piglets have less developed lymphoid follicles when compared with 6 month old pigs [79].

Tonsil dimensions vary with age and can range from 3.3 x 1.8 cm for pigs between 6 – 10 weeks old [76] to approximate 4.5 x 2 cm in adult animals [101]. Pig tonsils have been reported to have 160 to 190 oval to circular surface openings, corresponding to the entrances of tonsillar crypts [76]. Tonsillar crypts in pigs are covered by a non-keratinized squamous epithelium, supported by dense connective tissue and with a parenchyma uniformly populated with lymphocytes [103]. They are characterized by the presence of intraepithelial mucous cells, which suggests a possible role in the elimination of debris due to its secretory role [79]. Tonsillar lymphoid follicles are characterized by an oval shape with the long axis oriented perpendicular to the epithelial layer of crypts [79].

Studies show that during the development of tonsils in pigs, follicles are formed mainly by B-cells and T-cells present in the interfollicular area. IgM^+ cells are found in follicles of new born to 9 month old animals, while IgG^+ and IgA^+ cells are only seen after 4 weeks of age [104]. Activation of tonsillar T cell increases with age, and suggests a direct relation of levels of activation and differentiation of T cells with colonization of microbial flora. There is an association of microbial colonization with changes in the size and structure of follicles, which increased with the age, when compared pigs from birth to 4 weeks of age [104].

As can be seen in the above paragraphs, pig and human tonsils share many similarities and the possibilities to use pigs as translational models for studies involving tonsils are promising. The development of such important tissue as the tonsils involves many important features that are not the objective of this brief review. For readers interested in a more extensive review of pig and human tonsils, the articles by Horter et al [2] and Perry and White [53] are good sources.

MICROBIOME AND TONSILS

It was mentioned previously that in recent years there has been a bloom in microbiome publications in different sources using a sequencing approach. The study of the human microbiome has been led by a giant consortium of scientists that want to study the various aspects of the human microbiome, such as the structure, function and diversity in healthy populations and how disease correlates with changes in the microbiome [105, 106]. However, very few studies focus particularly on the tonsil

microbiome. Most available studies are focused on the oral cavity as a whole [107-110] or studied multiple body sites without a particular focus on the tonsils [111]. The only available study focused particularly on human tonsils analyzed the microbiota of tonsillar crypts in children and healthy adult patients diagnosed with tonsillar hyperplasia compared to patients with recurrent tonsillitis [112]. However there are no studies characterizing the development of the tonsil microbiome in humans, using a sequencing approach. Similarly, there are multiple studies focused on the microbiome of pigs in different body regions, particularly the intestinal microbiome, using a culture-independent approach. There are a few studies focused on the tonsillar microbiome of pigs [113-115] but none have studied the development of tonsillar microbiome of pigs. Use of a pig model as a model of human infants has been suggested, where pigs inoculated with human microbiota have the potential to simulate closely an overall human system behavior, especially because of the similarities shared between both species [116]. Further, it has been reported that 92% of the bacterial families identified in human tonsils are also present in pig tonsils [117], although different, often host-specific species are found. The following section will review what is known about the tonsillar microbiome of humans and pigs, focusing on the different methodologies used.

Human tonsillar microbiome: culture-dependent vs culture-independent approach. The initial studies characterizing the human tonsil microbiome were performed using a culture-dependent approach in order to establish the members of the tonsillar flora. Predominantly these studies were focused on characterizing the aerobic tonsillar bacterial population while a smaller number of studies have

characterized the anaerobic population. Most of these studies were based on young patients, mainly children between 1 and 13 years old (usually after tonsillectomy) [118-120], while a few others have also been done in older patients [121, 122].

Studies done in 1 - 3 years old children [118] as well as between 5 – 13 years [119], identified members of the genera *Streptococcus* and *Staphylococcus* as the main aerobic organisms isolated and members of genera *Bacteroides* and *Fusobacterium* as the predominant anaerobic organisms isolated. β-haemolytic *Streptococcus*, *Streptococcus pneumoniae* and *Haemophilus influenzae* were the prevalent aerobic bacteria [118, 121], followed by *Staphylococcus aureus* [121], while *Bacteroides fragilis* was the most common anaerobe isolated from the surface and core of tonsils of very young children [118]. *H influenzae* was most prevalent in youngsters vs adults and β-haemolytic *Streptococcus* was less prevalent in younger children (2-7) years, compared to older adults [121]. A predominance of anaerobic bacteria was found in adults compared to younger ages, identifying higher proportions of isolated anaerobes in adults (older than 15 years) vs younger children [121].

Using a culture-dependent approach, Kasenõmm et al. found mixed anaerobic and aerobic bacteria as core microorganisms in adult patients with acute tonsillitis [123]. The authors reported the frequent isolation of α- and β-haemolytic *Streptococcus*, *Staphylococcus aureus* and coagulase negative *Staphylococci*, and *Corynebacterium spp* as the aerobic population and members of *Peptostreptococcus*, *Propionibacterium*, *Actinomyces*, *Prevotella*, *Bacteroides* and *Fusobacterium* species as the most frequent anaerobes.

Although what is known about microbiome of tonsils by the culture-dependent approach is based on studies of samples of patients with acute tonsillitis, it has been reported that there is similarity in the organisms that form the tonsillar microbial flora of normal patients and patients with recurrent history of tonsillitis. However, a higher concentration of some bacterial members was present in the patients with tonsillitis. [119].

The use of culture-independent approaches to identify the tonsillar microbiome in humans has produced new insights. Using clone libraries of 16S rRNA genes, Aas et. al. studied the microbiome of 5 different patients/subjects in nine different oral locations including the tonsils [108]. They were able to identify that there is diversity in the bacteria identified in the study subjects where some bacterial species were found only in one subject, while others were present in all or most of the subjects. However, they emphasized that the tonsils had the highest numbers of different species compared to the other oral sites sampled.

Other studies have also examined the tonsillar microbiome, but using a 454 pyrosequencing approach. Liu et al [124] studied the composition of tonsillar bacterial microbiota in a 8 year old patient suffering chronic serous otitis media. The authors detected 9 different families in the tonsillar microbiome (*Pseudomonadaceae*, *Streptococcaceae*, *Fusobacteriaceae*, *Pasteurellaceae*, *Prevotellaceae*, *Flavobacteriaceae*, *Bacillales family XI*, *Carnobacteriaceae* and *Neisseriaceae*), and identified *Streptococcaceae* as the most dominant, with a relative abundance of approximately 69.2 %. Similarly, other authors have reported that multiple OTUs assigned to the genus *Streptococcus* were abundant in the tonsils, as well as others assigned to the genera

Prevotella, *Fusobacterium*, and *Neisseria* [111]. Some OTUs that could not be classified further than family level and belonging to the families *Neisseriaceae*, *Pasteurellaceae* and *Prevotellaceae* were also identified [111]. Segata et al [125] studied the microbial communities in ten different sites of the digestive tract (seven mouth surfaces, throat, palatine tonsils and colon (stools)), and they found that the community structure allowed them to group the samples in four distinct community types, where saliva, tongue, tonsils and throat formed one group. This group was characterized by a decreased relative abundance of the phylum *Firmicutes* and increased presence of members of the phyla *Bacteroidetes*, *Fusobacteria*, *Actinobacteria* and TM7. Also, members of the genera *Streptococcus*, *Veillonella*, *Prevotella*, *Neisseria*, *Fusobacterium*, *Actinomyces* and *Leptotrichia* were identified evenly distributed and present at an abundance of ≥2% on average, while members of the genus *Moraxella* were detected in low relative abundance. In contrast, Jensen et al [112], identified bacteria from different genera, including *Prevotella*, *Streptococcus*, *Haemophilus*, *Fusobacterium*, *Porphyromonas*, *Gemella*, *Neisseria*, *Veillonella*, *Capnocytophaga*, *Parvimonas*, *Rothia*, *Actinomyces* and *Treponema*, as the predominant members of the core microbiota in tonsillar crypts, which include both Gram (+) and Gram (-) bacteria, as well as aerobic and anaerobic organisms .

Finally, another approach used to investigate the components of the human tonsillar microbiome was the use of a selective isolation combined with the use of PCR denaturing gradient gel electrophoresis [126]. The authors profiled the microbiota from 10 healthy adults and identified three phyla as predominant in tonsils (*Firmicutes*,

Proteobacteria and *Fusobacteria*), and further identified members of the genus *Streptococcus* as abundant in tonsils.

Pig tonsillar microbiome: culture-dependent vs culture-independent approach. The majority of what is known about tonsillar microbiome of pigs based on culture-dependent approach studies is based on studies focused on identifying tonsillar bacterial isolates from specific genera. Devriese et al [127] studied the tonsillar bacterial population of pigs brought for necropsy suffering from a variety of conditions distinct from streptococcal infection, and yet isolated *Streptococcus suis* predominantly, followed by *S. dysgalactiae* and *Enterococcus faecalis.* In another study directed to identify the tonsillar Gram (+) flora of piglets before and after weaning, Baele et al [128] showed that the most prevalent bacterial genera found were *Streptococcus* (*Streptococcus suis*, *S. dysgalactiae*), as well as *Staphylococcus* (*S. hyicus*, *S. aureus*). *Arcanobacterium pyogenes* and *Actinomyces hyovaginalis* were also frequently isolated. The authors found marginal numbers of members of the genus *Lactobacillus* that appeared post-weaning. MacInnes et al [129] studied the prevalence of important respiratory bacterial pathogens in swine herds in Ontario, using a culture-dependent approach but also using serological and PCR-based tests, and identified a high prevalence of *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, and *S. suis* . O´Sullivan et al [130] identified the prevalence of porcine pathogens from pig tonsils collected at slaughter houses. This study determined that most prevalent bacteria isolated from pig tonsils were members of the genus *Streptococcus* (*S. suis*, *S. equisimilis*, *S. porcinus*), followed by *Arcanobacterium* (*Arcanobacterium pyogenes*), *Pasteurella* (*Pasteurella multocida*), *Staphylococcus* (*S. hyicus*, *S. aureus*), as

well as a lower prevalence of other pathogenic bacteria such as *Actinobacillus pleuropneumoniae*, *Yersinia enterocolitica*, *Haemophilus parasuis*, *Erysipelothrix spp*, *Listeria monocytogenes*, *Actinomyces sp* and *Salmonella spp*.. Finally, a culture-dependent approach to identify the microbial communities in pig tonsils, was done by Lowe et al [115] and by using aerobic cultivation, they isolated *Pasteurella multocida*, *Actinobacillus spp*., *Staphylococcus* (*S. aureus*, *S. epidermidis*), *Streptococcus* (*S. suis*, S. *dysgalactiae*), and *Escherichia coli* in half of the tonsil samples collected from two different herds. The same study also had a culture-independent approach using clone libraries of 16s rRNA genes. This study concluded that ~74% of the members of the microbial communities in pigs sampled from two different herds belonged to the families *Pasteurellaceae*, *Porphyromonadaceae*, *Bacteroidaceae* and *Prevotellaceae*. The authors reported members of the genus *Actinobacillus*, *Haemophilus*, *Pasteurella*, *Porphyromonas*, *Fusobacterium*, *Bacteroides*, and *Prevotella* as the dominant genera identified in both herds. It was highlighted from this study that although there were strong similarities between the herds, there were also unique genera that differentiated them.

Two other studies have been published using a culture-independent approach to identify the bacterial population in pig tonsils. Lowe et al [114] used a 454-pyrosequencing approach to identify the core microbial communities in tonsils, using samples from 18 – 20 week old pigs, which included the same samples from their previous study in 2011 [115] as well as new samples which were used to validate a non-invasive approach to collect samples to study the pig tonsillar communities, since the previous study involved the euthanasia of the animals and collection of tonsils. In this

study, the authors defined a core microbiome for pig tonsils [114]. This core microbiome was defined as composed of 5 phyla (out of 17 identified), which are in order of abundance *Proteobacteria*, *Firmicutes*, *Fusobacteria*, *Actinobacteria* and *Bacteroidetes*, which comprised ~98.8% of the identified organisms. At the family level, the authors identified that 8 families (out of 61 identified) comprised 90.4% of the members of the microbiome, and were in order of abundance *Pasteurellaceae*, *Moraxellaceae*, *Fusobacteriaceae*, *Veillonellaceae*, *Neisseriaceae*, *Peptostreptococcaceae*, *Enterobacteriaceae* and *Streptococcaceae.* Finally, at the genus level, 8 out of 101 genera formed the core microbiome and were identified as *Actinobacillus*, *Pasteurella*, *Alkanindiges*, *Fusobacterium*, *Haemophilus*, *Veillonella*, *Peptostreptococcus* and *Streptococcus*, comprising 85.1% of the identified genera.

The most recent identified study on the tonsillar microbiome of pigs was performed by Mann et al [113], who studied the metabolically active bacteria in tonsils from slaughter pigs and identified members of five phyla (*Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Spirochaetes* and *Fusobacterium*) as the most abundant in the sampled animals, comprising ~95.7 % of the identified phyla. Within those five phyla, members of 14 different genera had the highest relative abundance (*Prevotella*, *Porphyromonas*, *Campylobacter*, *Treponema*, *Streptococcus*, *Serratia*, *Paraprevotella*, *Bacteroides*, *Fusobacterium*, *Herbaspirillum*, *Flavitalea*, *Dehalospirillum*, *Alysiella* and *Pseudomonas*).

Taken together, the information summarized above allows us to conclude that there are multiple members of the microbiome shared between humans and pigs at phylum, family and even genus level. Further, there are multiple similarities shared between humans and pigs at the level of tonsil structure as well as the development of

the tonsils as lymphoid organs. This data leads us to believe that the pig can be an excellent translational model to study and test hypotheses about relationships between normal tonsillar microbiota and pathogenic bacteria in humans.

GOAL OF THIS THESIS

Prior research in the Mulks' laboratory developed and validated a non-invasive method to collect tonsil samples and used this method to describe the core tonsil microbiome in grower-finisher pigs [114]. One goal of this thesis project was to characterize the development of the tonsil microbiome in pigs from birth to market weight, by sequencing the 16s rRNA gene to classify or categorize the members of the microbiome and determine how those members change over time. Another goal was to use microscopy, both confocal laser and scanning electron microscopy, to examine the physical structure of tonsils and their attached microbial communities.

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CHAPTER 2. DEVELOPMENT OF THE TONSILLAR MICROBIOME IN PIGS FROM NEWBORN THROUGH WEANING

INTRODUCTION

Tonsils are lympho-epithelial tissues located at the junction of the oropharynx and nasopharynx that play a key role in surveillance of inhaled or ingested pathogens [1]. In pigs, the tonsils are colonized by numerous microbes, and serve as a reservoir for both pig pathogens and zoonotic pathogens with a high potential of transmission to humans [1-4]. Bacterial pathogens such as *Actinobacillus pleuropneumoniae, Streptococcus suis,* and *Salmonella enterica* are frequently found in tonsils of asymptomatic animals. Under conditions of stress, such as animal transport, these pathogens can spread to the lower respiratory and gastrointestinal tracts and be transmitted to other animals, including humans [5]. It has been suggested that the microbiome plays a preventive role in host colonization by pathogenic microorganisms [6-9] and also exerts regulatory roles in maintaining immune homeostasis, providing resistance to infection [10, 11].

In contrast to the rising number of studies characterizing the intestinal microbiome of mammals, there are few available studies characterizing the tonsillar microbiome and its development in those species. We have previously characterized the core microbiome of the tonsils in healthy 18-20 week old pigs [12]. In that study, members of the families *Pasteurellaceae, Moraxellaceae, Streptococcaceae, Fusobacteriaceae*, *Veillonellaceae, Enterobacteriaceae, Neisseriaceae,* and *Peptostreptococcaceae,* as well as the order *Clostridiales,* constituted the core tonsil microbiome in these grower-finisher pigs. Tonsils remain an under-explored habitat of the mammalian microbiome, and how the tonsil microbiome is established, how the structure of this community affects acquisition and

carriage of pathogens, and how it contributes to health and disease, in animals or in humans, are not well understood.

Currently, there are no studies following the development of tonsillar microbiomes in humans or in pigs, while other microbiomes, such as the intestinal microbiome in mammals, have been more broadly studied in the last decade. Studies conducted in pigs and humans have suggested that the development of the intestinal microbiome is a gradual and successional process [13-16] with a significant fluctuation after weaning [17]. The cessation of milk feeding as well as supplementation of other diets and feeding sources generate changes in the intestinal microbiota [18-22]. It is unknown if a similar successional process occurs in tonsils.

It has been shown for humans and pigs that in the development of intestinal microbial communities there is, initially, substantial individual variation in community composition that tends to shift over time, eventually becoming similar in the major phyla across individuals [15]. Environmental factors such as the maternal microbiota can play a relevant role in the initial development of the intestinal microbiota [13, 15, 23]. Nevertheless, the developing microbiome is composed of interacting bacteria and not simply randomly assembled microorganisms [14].

The goal of the current study was to utilize culture-independent, high-throughput sequencing of 16S rRNA genes to follow the development of the tonsillar microbial communities in pigs from birth through weaning.

MATERIAL AND METHODS

Animals. The Michigan State University Institutional Animal Care and Use Committee approved this study and the animal procedures. Ten crossbred sows (Yorkshire x Hampshire) from a high health status herd were used in this study. This herd has no history of recent respiratory disease and is considered free of *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, and Porcine Respiratory and Reproductive Syndrome virus by medical history. This herd experienced a recent outbreak of porcine epidemic diarrhea virus (PEDV), which was under control at the time this study was initiated. The herd has a history of vaccination against porcine circovirus type 2 (PCV2), erysipelas and atrophic rhinitis.

This herd was managed as a farrow to finishing facility with ~200 sows. Newborn piglets received a single intramuscular injection of Iron-Dextran. Piglets were weaned at three weeks of age (21 to 24 days – average weight 18 pounds) and moved to a nursery room, with litters maintained as pen mates. The weaned piglets were introduced to a solid diet based on a pellet ration (Pig 1300®, Akey Nutrition, Brookville, OH) supplemented with Carbadox® at a dose of 50 g/ton.

Individually identified sows of different parity, from primiparous (pregnant for the first time) to multiparous (pregnant multiple times), were purposely selected for this study and included two first parity sows (ear tags 1700 and 1707, respectively), one second parity sow (1631), one fifth parity sow (1445), one sixth parity sow (1402) and one tenth parity sow (1711). Four piglets from each of these six sows, selected randomly, were sampled within a period no longer than 8 hours post-birth (PB) (PB were piglets which might have interacted with other piglets or the sow before sampling) and then at 1, 2, 3 and 4 weeks of

age. An additional four freshly delivered piglets were randomly selected (N=16 piglets) from each of 4 crossbred Yorkshire x Hampshire sows (one second parity sow (1785), two third parity sows (1604, 1760) and one fourth parity sow (1704)), and sampled immediately at birth (AB) (AB piglets were sampled before they had any contact with external sources other than vaginal sources), avoiding any contact of the piglet with either the sow teats or the pen environment prior to sampling, with the purpose of determining the status of the microbiome right at birth. The litter from sow 1604 was sampled in 2014; the remaining three litters were sampled in 2015.

Collection of microbiome samples. Samples of the tonsil microbiome were collected from sows and piglets using either cytology brushes (Cytosoft™, Medical Packaging Corporation, Camarillo, CA) for very small piglets or tonsil brushes developed by our group and validated in previous studies [12]. Collection of samples was done by the same person, and as previously described [12]. Briefly, samples were collected at approximately the same time of day for each sampling time. Sow tonsillar samples were collected before they were fed. The pigs' movement was restricted either by holding them firmly wrapped with a towel, or by using a snare on larger animals. The mouth was held open with a mouth speculum while the tonsils were brushed. Right and left tonsils were brushed approximately ten times each, rotating the brush in a clockwise fashion. Brushes were removed from the pig's mouth and placed into a 50 ml sterile test tube containing 20 ml of 80% ice-cold ethanol. Samples were stored at -20 °C until processed.

The sow vaginal microbiome was sampled by introducing a sterile cotton swab approximately 8 cm into the vaginal tract and rubbing the vaginal walls with the swab while turning the swab in a clockwise fashion. The teat microbiome was also collected by using a

cotton swab and rubbing the teat surface of at least 10 teats per sow. Vaginal and teat swabs were placed individually into 50 ml sterile test tubes containing 20 ml of 80% ice-cold ethanol. Samples were stored at -20 °C until processed.

The sow fecal microbiome was sampled by collecting approximately 5 grams of feces directly from the rectum. Samples were placed individually into 50 ml sterile test tubes containing 20 ml of 80% ice-cold ethanol. Samples were stored at -20 °C until processed.

Isolation of community DNA. Sample extraction was performed as previously described [12]. Briefly, the 20 ml of 80% ice-cold ethanol containing the brushes, swabs or feces with the microbiome samples were thoroughly vortexed for one minute, divided into equal volumes and transferred to two sterile acid washed Corex® tubes, and centrifuged in a refrigerated Sorvall SS-34 rotor at 16,000 x g for 30 min. After centrifugation, the supernatant was removed and discarded. The pellet of one tube was suspended in 5 ml of ice-cold 80% ethanol and archived at -20 °C. The second pellet was suspended in 0.25 ml of phosphate buffered saline, pH 7, and transferred to PowerBead tubes (MoBio Laboratories, Carlsbad, CA) and vigorously shaken for approximately 2 min at room temperature using a MiniBeadBeater-16 (BioSpec Products, Inc., Bartlesville, OK). An exception to this protocol was followed with fecal samples, where both pellets were suspended in 0.5 ml of phosphate buffered saline, pH 7. Community DNA was then extracted using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer´s instructions. The concentration of extracted community DNA was determined by spectrophotometry, using a Nanodrop (Thermo Scientific, Wilmington, DE). Each sample was then split in two vials; one was archived at -80 °C and the other was processed for sequencing.

Illumina sequencing and sequence analysis. For Illumina sequencing, samples were processed at the Michigan State University Research Technology Support Facility (RTSF) using an Illumina MiSeq platform. Negative controls consisting of either DNA-free water or MoBio C6 solution ("blank library controls", [24]) and positive controls consisting of either *Escherichia coli* DH5α genomic DNA or a well-characterized activated sludge polymicrobial community [25] were included in the sequencing runs. Briefly, the V4 region of the 16S rRNA gene of the community DNA was amplified using uniquely indexed primers for each sample, as described by Caporaso [26]. After PCR, amplification products were normalized using an Invitrogen SequalPrep normalization plate. The normalized samples were pooled and PCR reaction cleanup was done with AMPure XP beads. After quality control and quantitation, the pool was loaded on an Illumina MiSeq v2 flow cell and sequenced with a 500-cycle v2 reagent kit (PE250 reads). Base calling was performed by Illumina Real Time Analysis Software (RTA) v1.18.54 and output of RTA demultiplexed and converted to FastQ files with Illumina Bcl2fastq v1.8.4.

Amplicon analysis was performed using the open-source, platform-independent, community-supported software program mothur v.1.38.0 (http://www.mothur.org) [27]. Processing of the raw sequencing data was done according to the mothur standard operating procedure (http://www.mothur.org/wiki/MiSeq_SOP) [28]. Alignment was accomplished using the mothur-formatted version 123 of Silva 16S rRNA gene database [29]. Sequences were classified and any sequences classified as Chloroplast, Mitochondria, unknown, Archaea, or Eukaryota were removed from the data set. Subsampling at 5907 sequences per sample was performed, followed by a preclustering of the sequences and removal of chimeric sequences using a mothur-formatted version of the Ribosomal Database Project (RDP)

training set version 14 and uchime, based on the mothur protocol. Sequences were classified into Operational Taxonomic Units (OTUs) of ≥97% sequence identity. Singleton and doubleton reads were removed, followed by subsampling at 3776 sequences per sample. Because the negative controls consistently showed high levels of contaminants that skewed the results seen with some of the low biomass samples such as those from the newborn piglets, especially those from Litter 1700, 4 OTUs (*Ralstonia*, *Bacillaceae 1*, *Burkholderia,* and *Brevundimonas*) were also removed from the data set prior to final analysis. A SIMPER comparison of Litter 1700 piglet and sow samples to the relevant negative controls showed 66.1% or greater dissimilarity between the negative controls and the pig samples, rejecting the hypothesis that the sequence data for Litter and sow 1700 samples is due to contamination only [24]. Therefore these samples were included in the final data set. For the final analysis of the data, samples were subsampled to 1979 reads per sample. The full data set analyzed is available as a supplemental file at (https://figshare.com/s/1ac201b155d662dcc646).

Diversity and statistical analysis. The statistical analysis was performed using a clustering cutoff of 3% for the processed sequences. Mothur output files were used to estimate diversity indexes and core microbiomes. PAST3 was used for generation of the UPGMA dendogram file, SIMPER, coordinate analysis of the samples, two dimensional scatter plot and 95% concentration ellipses [30]. ImageJ was used to measure the area of the ellipses for the two dimensional scatter plot [31]. Dendogram construction was performed using FigTree v.1.4.2. (http://tree.bio.ed.ac.uk/software/figtree/). RStudio (Version 0.99.446; https://www.rstudio.com/) and libraries: gplots (https://CRAN.R-project.org/package=gplots), plot3D (https://CRAN.R-project.org/package=plot3D), and rgl

(https://CRAN.R-project.org/package=rgl) were used to generate heatmaps and tridimensional scatter plots, respectively. Inkscape 0.91 (https://inkscape.org/en/download/mac-os/), was used to process images and edit labels.

Availability of supporting data. Raw sequence data is available at the NCBI database (SRA accession number: SRP110992) and the code for the mothur analysis is available at (https://figshare.com/s/2c98593a953cc9bb1366).

RESULTS

A total of 171 samples derived from the tonsillar microbiome of piglets and sow tonsils, teat skin, vagina and feces as well as control samples were processed. One hundred forty-four pig samples were collected in Spring 2014, and an additional 12 pig samples were collected in Fall 2015. A total of 26 samples with less than 3776 final reads after singleton and doubleton removal, were not included in the final analysis. One of the approaches that we used was comparative analysis of core microbiomes. We defined the core microbiome for a specific litter as the OTU members of the microbiome that were present in at least 75% of piglets of a litter, when litters had at least 4 samples; otherwise core OTUs were defined as OTUs present in all the samples. For the sow samples, where 6 samples were analyzed, we considered OTUs present in 66.6% of the samples to be core OTUs. Further, core OTUs were defined as present in a minimum relative abundance equal or higher than 1% and/or 0.1% of total reads for a selected litter and/or period (Table 2.2.).

The tonsillar microbiome found in PB samples clusters by litter. Among the 156 samples collected, 40 samples contain the tonsillar microbiome of newborn piglets (Table 2.1.). Twenty four of these 40 samples were collected from PB piglets and the remaining 16 samples were collected from AB piglets. Six samples were discarded from the analysis due to low number of reads.

Table 2.1. Identification of sows, litters, sample collection and times of collection

					Newborns	Sow samples			
Sow ID	# sow parity	Litter members	# piglets	# analyzed	Sampling	Feces	Teats	Tonsils	Vaginal
1700	1	10,11,12,13	4	4	PB	1	1	1	1
1707	1	31,32,34,35	4	3	PB	0	1	1	1
1631	2	15,16,17,18	4	4	PB	0	1	1	1
1445	5	36,39,40,42	4	4	PB	0	1	1	1
1402	6	1,2,4,6	4	3	PB	0	1	1	1
1711	10	22,23,24,26	4	4	PB	1	1	1	1
1785	2	49,50,51,52	4	3	AB	0	0	0	0
1704	4	66,67,68,69	4	4	AB	0	0	0	0
1760	3	79,80,84,85	4	3	AB	0	0	0	0
1604	3	43,44,45,46	4	2	AB	0	0	0	0
Total			40	34		2	6	6	6

PB. Samples were collected from newborn piglets in a period not greater than 8 hours post birth.
AB. Samples were collected from newborn piglets immediately after birth.

A dendrogram clustering of PB piglet samples and sow derived samples, based on the Bray-Curtis dissimilarity index (Figure 2.1.) showed that samples from each litter clustered together with littermates. Microbiomes derived from piglets from primiparous sows (Litter 1700 and 1707) clustered in separated clades, as did the piglets from sow 1402. In general, PB piglet samples were more closely associated with samples derived from the sow teat microbiome as opposed to other sow samples. However, piglets from litter 1700 also clustered with the sample from the sow vaginal microbiome.

Samples from the sow tonsils and sow feces clustered together by source. Samples from the vaginal tract of multiparous sows clustered together, but were distinct from those from the two primiparous sows.

A heat-map representation of the major OTUs reveals the differences driving the clustering (Figure 2.2.). In the PB piglets from sows 1445, 1631, and 1711, all multiparous sows, the most abundant OTUs were *Pasteurellaceae, Streptococcus*, *Moraxella, Rothia,* and *Staphylococcus* (OTUs 001, 002, 003, 007, and 009, respectively). PB piglets from sow 1402

(parity 6) contained significant numbers of *Pasteurellaceae, Streptococcus*, and *Moraxella* (OTUs 001, 002, and 003) but also contained large numbers of *Enterobacteriaceae* (OTU005) and *Clostridium sensu strictu* (OTU010). In contrast, piglets derived from primiparous sows 1700 and 1707 had a very low abundance of *Pasteurellaceae*, *Streptococcus*, and *Moraxella* (OTUs 001, 002 and 003), but a high abundance of *Staphylococcus* (OTU009) as well as several anaerobic organisms.

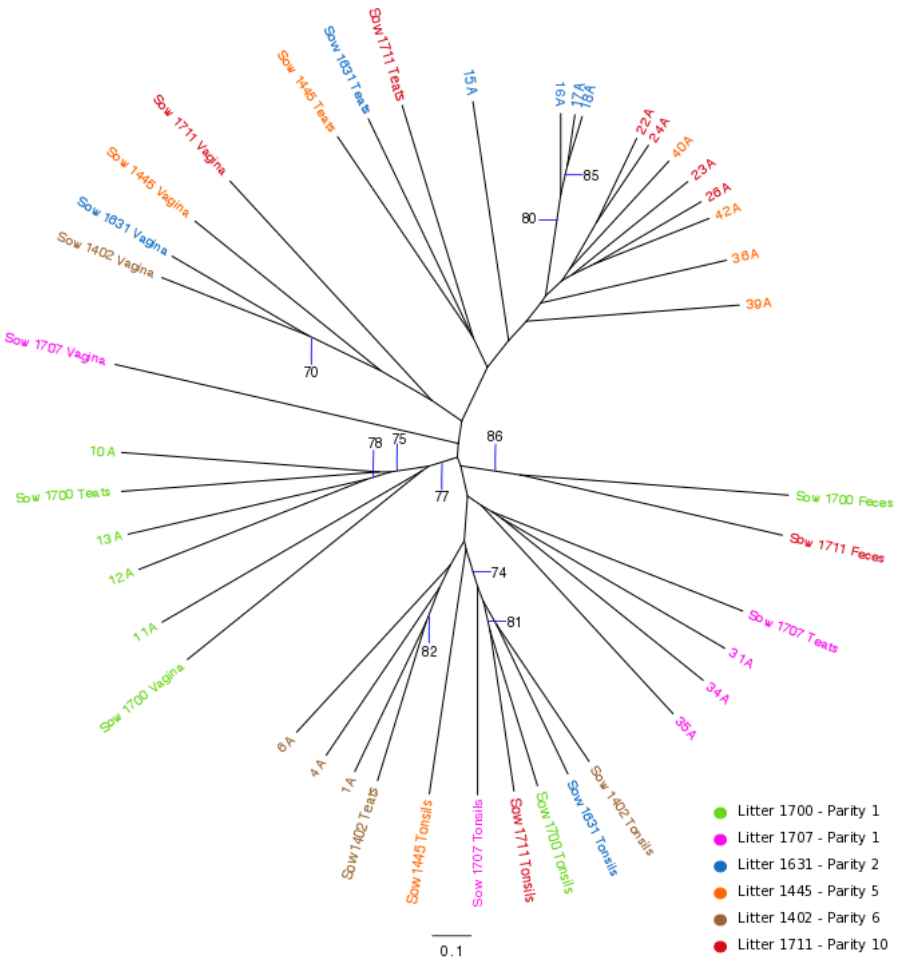

Figure 2.1. Unrooted Bray-Curtis dendrogram of PB and sow microbiomes. The samples are color coded by the source. Bootstrap values higher than 70 % at 1000 iterations are shown.

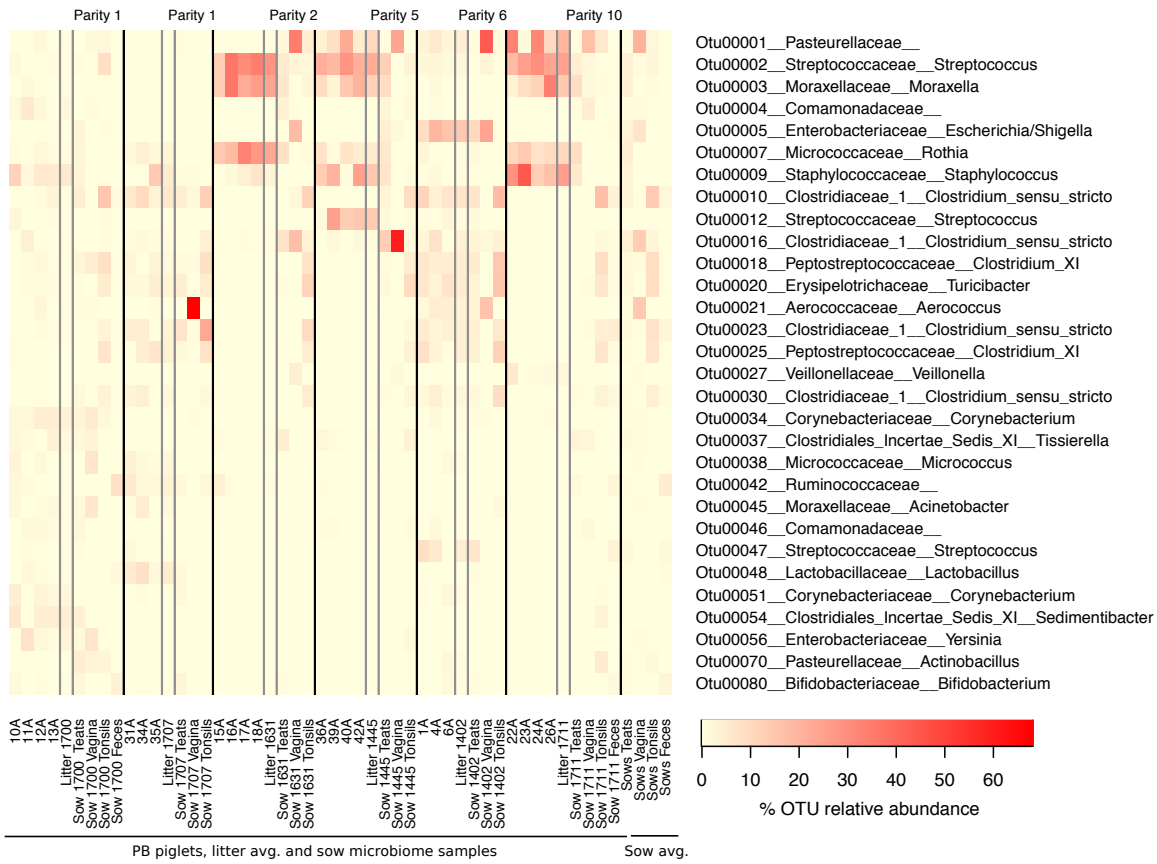

Figure 2.2. Thirty most abundant Operational Taxonomic Units (OTUs) for piglets and sow samples. Heat-map showing the relative abundance of the top 30 OTUs identified for all samples. The figure shows the relative abundance of the OTUs for each PB piglet and sow samples, as well as the average for the litters and sow samples.

A significant proportion of the microbiome of the piglets came from maternal sources. We processed samples derived from teat skin, vagina and tonsil from six sows belonging to the PB group (Table 2.1.), and fecal samples from two of these sows. We compared and traced which OTUs were identified as core microbiome for each litter and if they were identified as core for microbiomes derived from the different sow sources (Table 2.2).

The OTUs identified as core for PB litters varied between litters. As was seen in Figure 2.2., *Pasteurellaceae*, *Streptococcus* and *Moraxella* (OTUs 001, 002 and 003) were identified as core with a minimum relative abundance equal or higher than 1% and were found in high proportions in most piglets from multiparous sows but not piglets from primiparous sows. When sow sources for these three OTUs were examined, OTU001 *(Pasteurellaceae)* was identified as a core organism in the sow vaginal tract, representing on average 20.1% of the vaginal microbiome. However, OTU001 was present in only low amounts in the vaginal tracts from primiparous sows. In contrast, OTU002 (*Streptococcus)* was found in both vaginal and teat skin samples, while OTU003 (*Moraxella*) was found mainly in teat skin samples; both were more prevalent in samples from multiparous sows. In addition, *Rothia* (OTU007) and *Staphylococcus* (OTU009) were identified in high proportions and as core for most PB litters regardless of sow parity, as well as core for the sow teat microbiome, but not as part of the core microbiome for other sow samples.

These results suggested that the PB piglets had acquired new organisms from teat skin or other sources within the first few hours of life. To test this hypothesis, we collected samples from piglets immediately at birth (AB), prior to any contact with sources other than the uterus and the sow vaginal tract. These samples were very sparse in content, and unfortunately the resulting sequence data was dominated by organisms known to be frequent contaminants in DNA extraction kits and library preparation kits, including *Comamonadaceae*, *Sphingomonadaceae* and *Xanthomonadaceae* [24]. However, when these likely contaminants were deleted from the data, it was clear that the organisms most frequently seen were organisms associated mainly with the sow vaginal tract, including OTU002 *Streptococcus*, *Corynebacterium* (OTU034 and 051), and several anaerobic

organisms including multiple OTUs of *Clostridiaceae and Peptostreptococcaceae.* OTU001 (*Pasteurellaceae*) was also found although in low numbers.

An analysis of the microbiome derived from sow sources as well as from PB piglets at the family level showing the 20 most abundant taxa is presented in Figure 2.3. These taxa generally include multiple OTUs. *Streptococcaceae* (20 OTUs), *Staphylococcaceae* (4 OTUs), *Micrococcaceae* (10 OTUs), *Pasteurellaceae* (21 OTUs) *Moraxellaceae* (29 OTUs) and families belonging to the *Clostridiales* (746 OTUs) were present in high abundance in the PB piglets, and were also found in the sow vagina samples and/or the sow teat skin samples.

Tonsil communities of newborn piglets differed initially between litters but by three weeks of age clustered together reflecting similar composition. We inquired if, over time, the tonsillar communities of piglets reached a common microbiome. A principal coordinate analysis (PCoA) based on Bray-Curtis distances for the PB newborn through week 4 samples (Figure 2.4.A. and 2.4.B.) showed a dynamic pattern in the samples. The PB newborns were widely spread although two distinct clusters were detectable. Over the next three weeks the microbiomes formed increasingly tighter clusters reflecting greater similarity as the animals aged. However, in the fourth week there was a dramatic shift, where, instead of continuing to cluster together more tightly as observed in previous weeks, samples were scattered.

Further analysis using a SIMPER approach based on Bray-Curtis distances (Table 2.3.) reinforced what we observed in the PCoA plots. Microbiome samples from PB piglets were dissimilar when compared with others and the value of dissimilarity fluctuated, with the lowest dissimilarity being 45.8% for litter 1445 vs 1711 and the highest being 94.04% for litter 1631 vs 1707. Although there was a substantial variability in the overall dissimilarity

between litters, as time advanced the dissimilarity value between litters decreased and reached the lowest values in the third week, when values were as low as 23.5 % for litters 1402 vs 1707 and the highest was 37.3% for litters 1402 vs 1445.

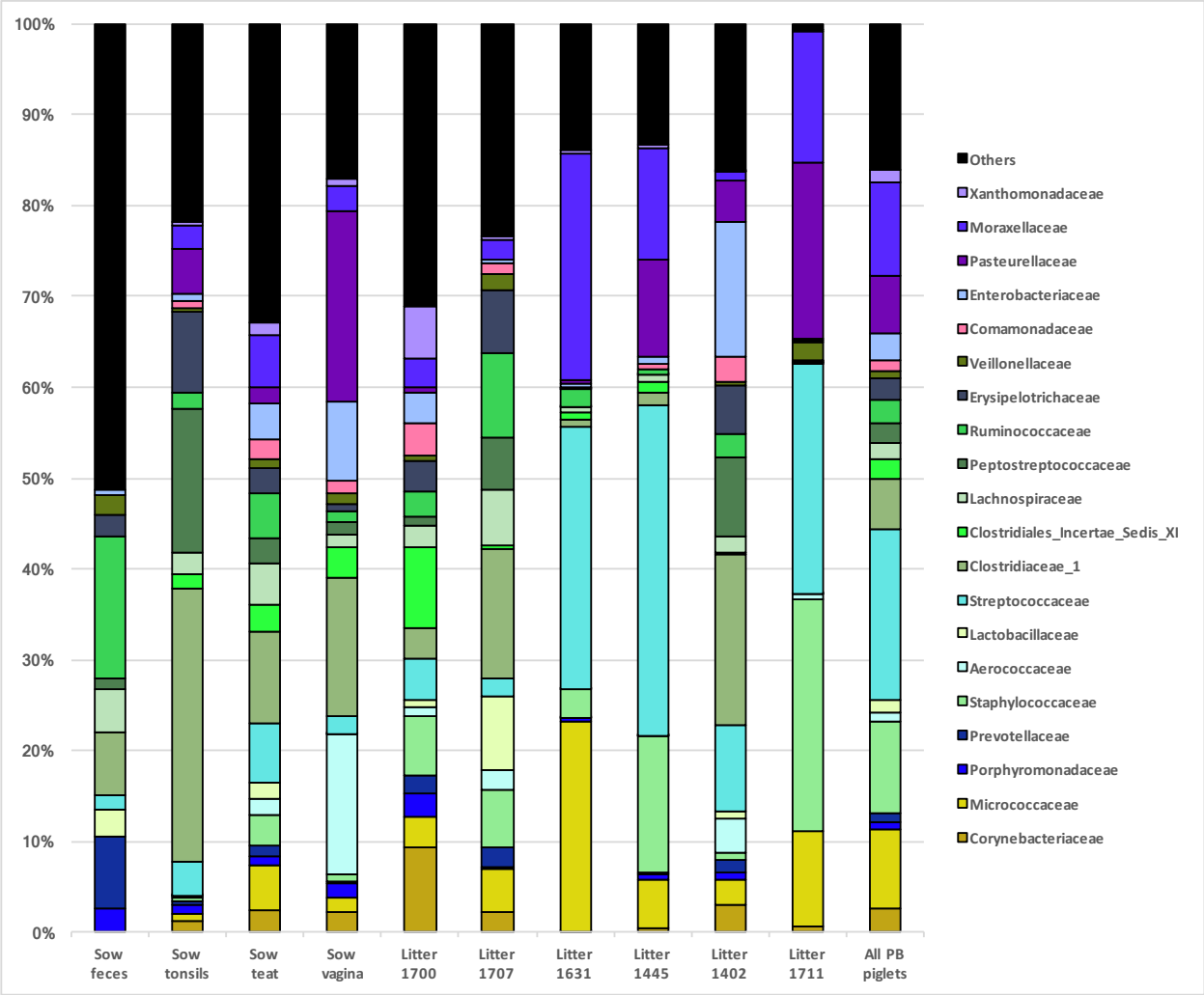

Figure 2.3. Twenty most abundant families identified in sows and PB microbiome samples. Bar plot shows the mean values for each family in Sow and PB samples, including each PB litter (percent of total OTUs). "Others" represent members of bacterial families different from the 20 most abundant families identified.

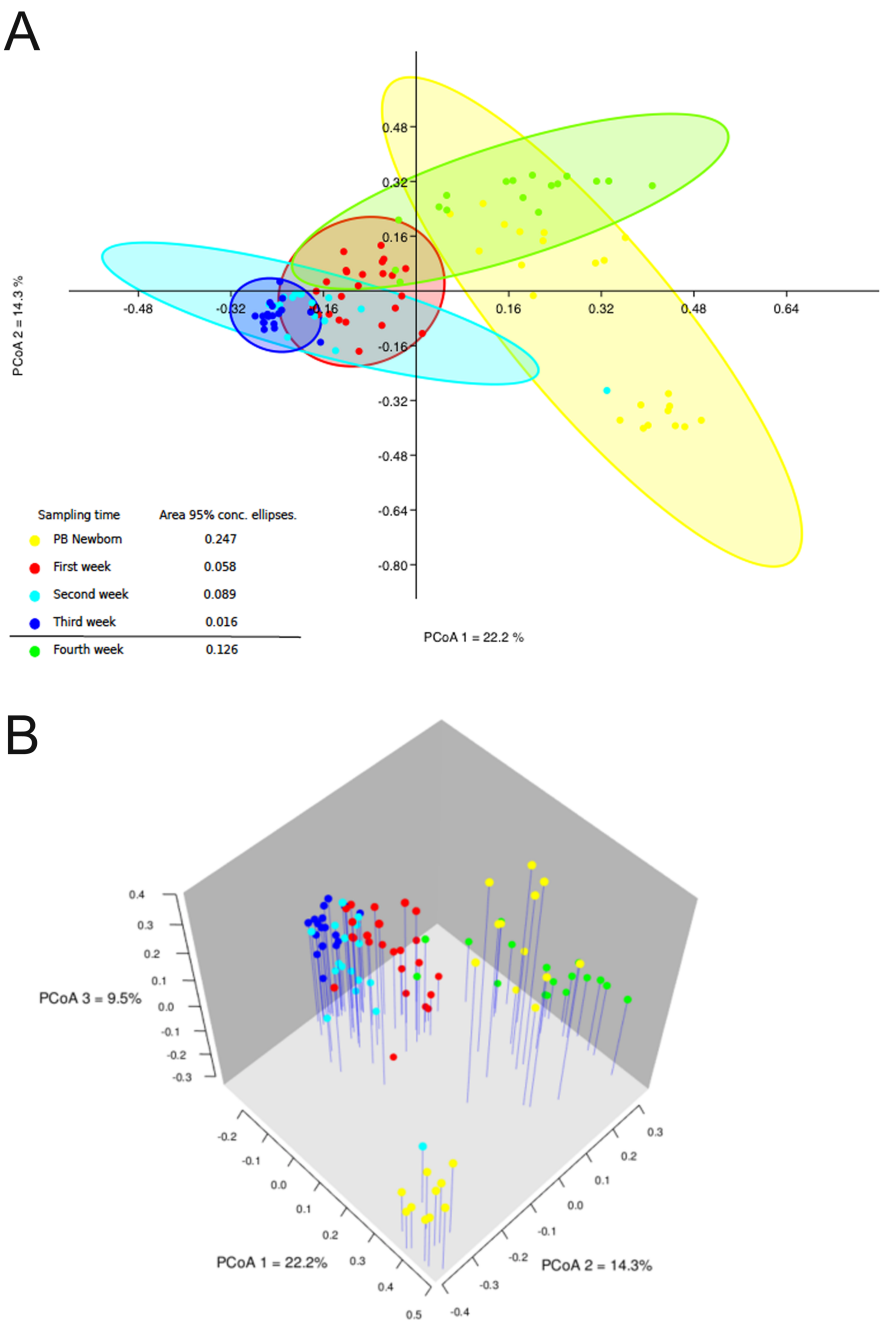

Figure 2.4. Principal Coordinate Analysis (PCoA) characterizing the tonsillar microbiome from PB piglets through the different sampling times. Two dimensional plotting illustrating the distribution of microbiome in the first two axes, the 95%

Figure 2.4. (**cont´d).** concentration ellipses for newborn piglets through four weeks and the relative area for the ellipses (A). Three-dimensional plot illustrating the main three axes for the distribution of the microbiome of PB piglets aged newborn through four weeks (B).

To visualize the temporal patterns of distribution of different bacterial families, we charted the mean value for the 20 most commonly identified families per sampling time (Figure 2.5.). While there were litter to litter variations in the microbiome of PB newborn piglets, as shown in Figure 3, the differences between litters disappeared through the successional development of the microbiome in the following weeks.

Members of the families *Micrococcaceae* and *Staphylococcaceae* were abundant in PB newborns but decreased drastically and almost disappeared in the following weeks. By 1 week of age, the tonsil microbiome was dominated by members of the *Pasteurellaceae*, *Moraxellaceae*, and *Streptococcaceae* families, a dominance that remained throughout the lives of these pigs (unpublished data). In contrast, some members of the microbiome appeared and disappeared over time, such as *Porphyromonadaceae* and *Flavobacteriaceae* which appeared at weeks 1 to 3, as well as *Fusobacteriaceae* and *Leptotrichiaceae* that were present only during weeks 2 and 3.

The transition between third and fourth week represents a critical period for the development of the microbiome. According to MSU farm management practices, piglets were weaned between the third and fourth week. We investigated if the management practices experienced by the piglets (weaning, shift to a nursery room and introduction to a pelleted ration supplemented with Carbadox®), correlated with the timing of shifts in community structures.

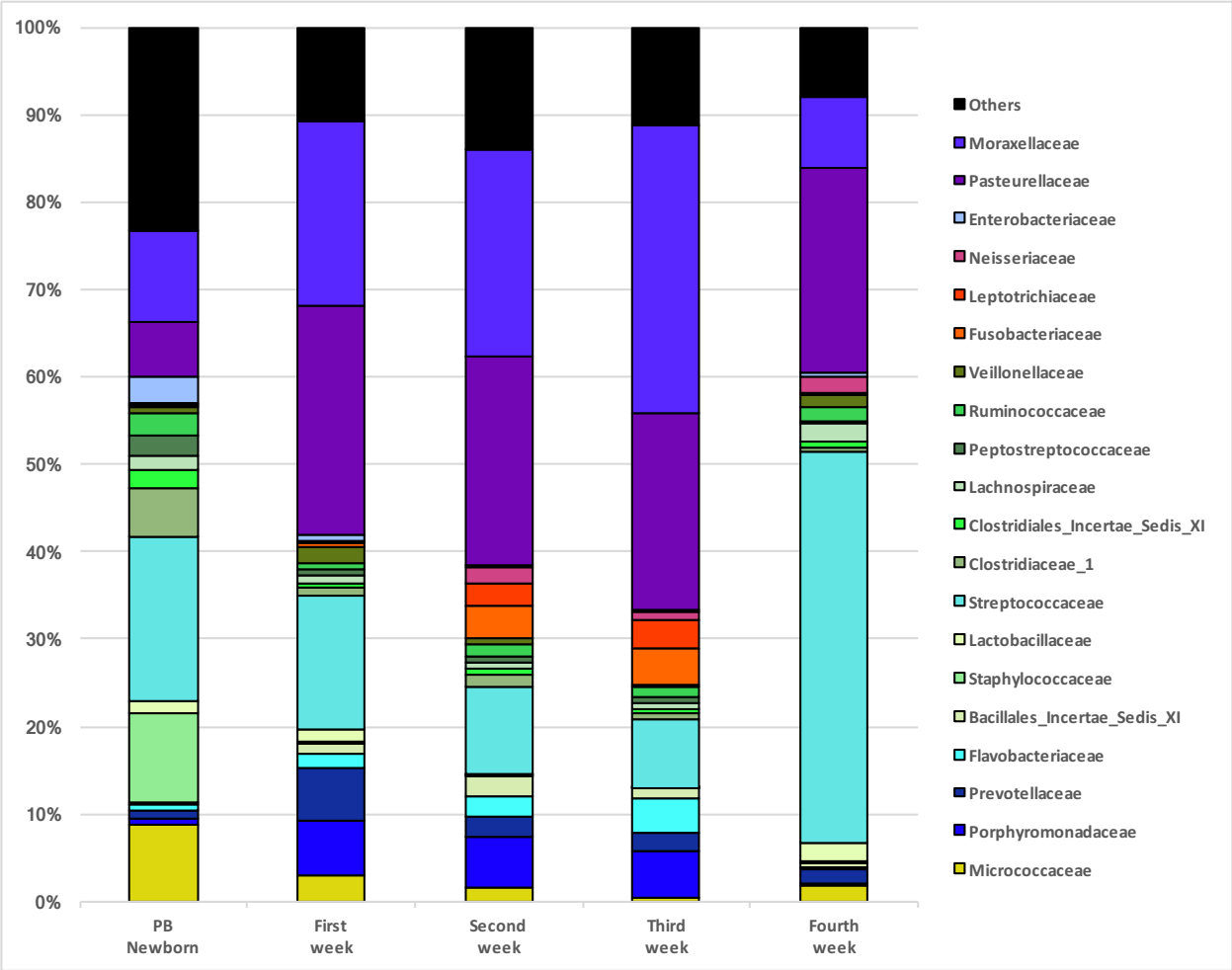

Figure 2.5. The abundance of the twenty most common families in PB piglets sampled from newborn through four weeks. Bar plot shows the mean values for the twenty most abundant families identified over the first four weeks of life (percent of total OTUs).

An analysis of the members of core microbiome (Table 2.4. and Table 2.5.) demonstrated that for the third week, OTU001 (*Pasteurellaceae*), OTU002 (*Streptococcus*), OTU003 (*Moraxella*), OTU006 (*Porphyromonas*), OTU017 (*Fusobacterium*) and OTU026 (*Flavobacteriaceae*), identified as core with a minimum relative abundance equal or higher

than 1%, were shared among all the litters, and represented on average 70.1% of the identified OTUs. In addition, OTU012 (*Streptococcus*), OTU15 (*Prevotellaceae*), OTU018 (*Peptostreptococcaceae*) and OTU024 (*Bacillales Incertae Sedis XI*) were core members at a minimum relative abundance of 0.1% or higher. OTU001 (*Pasteurellaceae*), OTU002 (*Streptococcus*) and OTU003 (*Moraxella*) dominated the microbiome, together representing on average 59.5% of the identified OTUs. An analysis at the family level (Figure 2.5.) for the same period shows the families *Moraxellaceae* and *Pasteurellaceae* as the most abundant families with an abundance of almost 55.6%, followed by members of the families *Streptococcaceae*, *Porphyromonadaceae*, *Fusobacteriaceae*, *Leptotrichiaceae* and *Flavobacteriaceae*, together with an abundance of 24.5%.

The fourth week showed a different panorama, where only two OTUs, OTU001 (*Pasteurellaceae*) and OTU002 (*Streptococcus*), were identified as core at 1% for all the litters. However, in contrast to the third week, these two OTUs represented on average 67.6% of the identified OTUs. In the fourth week, members of the *Streptococcaceae* family increased dramatically 5-6 fold compared to week 3, *Pasteurellaceae* remained at the same levels, and members of *Moraxellaceae* decreased 4 fold. Members of *Porphyromonadaceae*, *Flavobacteriaceae*, *Fusobacteriaceae*, and *Leptotrichiaceae* decreased to negligible numbers in week 4.

Furthermore, a SIMPER analysis (Table 2.3.) indicates that the dissimilarity in percentages between litters showed changes; instead of continuing the downward trend as observed in previous weeks, the dissimilarity values generally increased by 10-20%, with the lowest being 32.04% between litters 1631 and 1402 and the highest 47.98 % between 1700 and 1445.

DISCUSSION

In this study, we followed the development of the tonsillar microbial communities in pigs from birth through weaning. We focused on identification of the source of bacteria found in the tonsils, the successional development, and the apparent effect of weaning on the tonsillar microbiome.

Analysis of the microbiome in the PB piglets showed that the most abundant organisms were *Streptococcus*, *Staphylococcus, Moraxella, Rothia,* and *Pasteurellaceae* (OTUs 002, 009, 003, 007, and 001 respectively). Based on comparative analysis of putative source microbiomes and piglet tonsil microbiomes, as well as cultivation studies, we concluded that while the *Pasteurellaceae* and *Streptococcus* were most likely acquired from the sow vaginal tract during the birth process, *Moraxella, Staphylococcus* and *Rothia* were likely acquired from the sow teat skin (or milk, which we did not sample) within a few hours after birth.

To determine what organisms were actually acquired during birth, we collected samples from piglets immediately after they were born (AB piglets), prior to contact with teat skin or other sources except the sow vaginal tract. As described in results, these samples were very sparse and the microbiome identified in them was heavily biased by likely contaminants from either the DNA extraction kit or library construction kit used. This has recently become a recognized problem in microbiome studies on low microbial biomass samples [24, 32, 33]. While we ran appropriate positive controls and no template library construction controls, we did not run DNA extraction controls with the same kit used in this study. We have done this with a different lot number of the same kit, and used the

information from that experiment as well as other published studies [24] to help analyze the data on the microbiome in AB piglets.

When we removed what we considered to be contaminants from the data set for AB piglets, the remaining organisms included OTU002 *Streptococcus* and OTU001 *Pasteurellaceae*, both of which colonized, multiplied, and persisted for the life of these pigs. It should be noted that we have cultured *Streptococcus suis* from at least half of these AB piglet samples, as well as from sow vaginal samples, providing additional support for the conclusion that this organism is acquired during the birth process. We also identified organisms such as OTU016 *Clostridium sensu strictu* and two OTUs (034 and 051) identified as *Corynebacterium* that were found in the sow vaginal tract and in AB and PB piglets, but not older piglets, demonstrating that some of the organisms acquired during birth do not persist in the tonsils.

If these organisms failed to adhere to the tonsil epithelium, they would be washed out of the oropharynx, and other organisms acquired from the vaginal tract, such as *Streptococcus*, which do adhere to the tonsil epithelium, would be able to colonize and multiply. Further, once the piglets had contact with other sow sources such as the teat skin or milk, new colonizers such as *Staphylococcus* could be acquired, leading to a sequential development of the tonsil microbiome starting within the first few hours of life. The clustering pattern observed in Figure 2.1., where samples from three of the four multiparous litters clustered together as a group with sow teat skin samples and litters born from primiparous sows clustered with the cognate teat and vaginal or just teat samples, reinforce that teat and/or vaginal samples from sow are the initial source of the microbiome for PB newborns. Our findings are supported by the results of other studies, such as the study of

Mandar and Mikelsaar [34], which characterized the initial colonization of the external ear canal in newborn humans and compared the microorganisms found with the vaginal flora of their mothers and concluded that there is a significant influence of the vaginal microflora in the initial microbial population found in the newborns. Further, Bokulich et al [13], studying the effect of antibiotics, birthmode and diet in the development of fecal microbiota in children during early life, suggested that early colonizers are transmitted to children from maternal microbiota. They found that children delivered vaginally shared more OTUs derived from the vagina than children delivered by caesarean. Additionally, they identified that the infant fecal microbiota was initially associated with vaginal and rectal maternal microbiota, but later was more associated with maternal fecal microbiota. Further, a study of the development of the human intestinal microbiome suggested that the bacterial population detected in human infants in early stages of life might be determined by specific bacteria to which infant was previously exposed, based on similarity patterns observed between infant samples and maternal sources as breast milk and vaginal swabs [15]. These authors identified *Streptococcus* and *Staphylococcus* as well as other aerobes as first colonizers [15]. Similarly, we observed that piglets sampled within short period after being born had high proportions of *Streptococcus*, *Moraxella* and *Staphylococcus* in their tonsils.

Our results demonstrated a strong litter effect in the tonsillar microbiome in PB piglets (Figure 2.3.). However, over the following three weeks there was a gradual successional development in the tonsil microbiomes of all piglets and by the third week the microbiomes of all piglets from all litters were highly similar (Figure 2.4.). This was true even for a litter that did not share the same farrowing room, as was the case for piglets from litter 1445 (data not shown). This is in contrast to the reported development of the pig

intestinal microbiome over the first few weeks of life, where no obvious effect of litter was seen [35].

In this successional development, some organisms such as *Staphylococcus* and *Micrococcaceae* that were found in high proportions in the PB piglets decreased dramatically within the next 2 weeks, suggesting a role only as initial weak colonizers. These organisms are commonly isolated from multiple skin locations [36, 37] and vagina [38] and are likely to be vertically transmitted from sow to offspring. Over the same period there was a concomitant increase in stronger colonizers, particularly members of the families *Pasteurellaceae* and *Moraxellaceae*, as well as maintenance of the levels of *Streptococcaceae*. These three families, which contain both commensals and pathogens that are residents of mucosal surfaces of animals and humans, comprise a large proportion of the tonsil microbiome throughout the lives of pigs. Members of the order *Clostridiales* were also identified as a small but consistent part of the microbiome throughout the first 3 weeks, which is not surprising since they were identified as part of the core tonsillar microbiome of pigs [12] as well as members of intestinal microbiome [16, 19, 35, 39].

Over this period we also found several transitory OTUs or families that appeared and disappeared at specific time points. For example, *Porphyromonadaceae, Prevotellaceae,* and *Flavobacteriaceae* appeared at week 1 and increased slightly over the next 2 weeks. Similarly*, Fusobacteriaceae* and *Leptotrichiaceae* appeared at weeks 2 and 3. By week three, this successional development of the tonsil microbiome of all of the piglets, regardless of litter, led to a distinct common consortium of bacterial species.

Similarly, Palmer et al [15], studied the microbiome profiles from human infant stool samples and suggested that although initially the microbiome was very distinct between

individuals, over time it converged towards a common profile. The authors followed the development of the intestinal microbiome in 14 human infants, and showed that there was a considerable variation in the colonization process among individuals. Each infant had a distinct arrangement of bacterial species that it acquired and maintained. The acquired microorganisms had a temporal pattern in which they appeared and disappeared; however, they reached a stable population over time, with some taxonomic groups persisting while the presence of other taxa was only transient [15]. The authors reported the occurrence of significant shifts in the population assembly, which seemed to stabilize over time.

At 3 weeks of age, the piglets were weaned onto a solid ration containing the growth promoter Carbadox® and were moved from the farrowing room to the nursery, while kept in groups with their littermates. Comparison of the microbiome composition for the third and fourth weeks showed a major shift associated with the significant stress event of weaning, with its environmental, social and feed changes. Our results are in concordance with other studies following the development of the intestinal microbiome in humans and pigs, which reported significant changes in microbiome composition associated with life events [14, 15]. In pigs, it has been demonstrated that the transition from nursery to weaning is associated with a significant change in the intestinal microbiota [19, 40]. However, the effect of this transition on the tonsillar microbiome has not previously been studied in pigs or humans. In this study, we have demonstrated that the transition from farrowing to a nursery room in parallel with weaning and supplementation of the diet with Carbadox® was associated with a major shift in the tonsil microbiota.

The most obvious effect of weaning was the 5-6 fold increase in members of the *Streptococcaceae*, from an average of 8% before weaning to ~43% of the total identified

families after weaning. These organisms were primarily members of the genus *Streptococcus*. There was a concomitant decrease for members of the *Moraxellaceae* family, which decreased approximately 4-fold, from ~32% before weaning to ~8% after weaning. In addition, *Fusobacteriaceae*, *Leptotrichiaceae*, *Porphyromonadaceae*, and *Flavobacteriaceae* decreased dramatically. However, at the same time, the proportion represented by *Pasteurellaceae* remained constant.

It is not clear whether a single stress, such as change in food or change in environment or application of antibiotic, or a combination of stresses was responsible for the major disruption in the tonsil microbiome at this time. It has been demonstrated in multiple studies that the intestinal microbiota composition was deeply perturbed when the host was treated with antibiotics [8, 13, 15, 41-44]. In our study, the piglets were supplemented with Carbadox® in food at the time of weaning. It was reported by Looft et al [41] that the structure and composition of the intestinal community of pigs supplemented with Carbadox® changed significantly, where the relative abundance of *Prevotella* increased associated with Carbadox® administration as a result of decreased abundance of other bacteria. Another study correlating changes in microbiota with changes of diet during nursing and weaning found that the fecal population of *Prevotellaceae* increased ~50-fold in weaned pigs compared with nursing animals [19]. In our study, members of *Prevotellaceae*, a minor population in the tonsils, decreased slightly from 2.7 % for week three to 1.9% for week four. We can only speculate about the opposite results identified in our studies, since we are comparing different niches (feces vs tonsils) and we did not have controls that were not fed Carbadox® as this was not a goal of our study.

It has been suggested that the environment also plays a relevant role in the initial acquisition of the microbiome. Mulder et al [11], followed the development of gut microbiota and the potential impacts of early environmental changes, and demonstrated a major impact in the microbial diversity related with those changes and that the impact of those changes are preserved through adulthood. One of the major changes experienced by the weaned piglets is the separation from the sow and the introduction to new food. A deep impact in the intestinal microbial composition has been seen associated with cessation of breast feeding and introduction to a different diet [20].

We observed members of the microbiota that were present all the time, some in high relative abundance while others were found in low abundance. Similarly, there were also transient members whose exact role in the development of the tonsil microbiome is unclear but worth investigating in future studies. It is possible that these transient organisms appeared as secondary colonizers but were displaced by other members of the microbiota, or that these microorganisms were adversely affected by the stressful event of weaning and/or the Carbadox® supplementation and thus disappeared. We have an imprecise idea about the true role of Carbadox® in the development of the tonsillar microbiome, since our study was not specifically intended to answer this question. We unfortunately did not collect sow milk samples, or samples from the pen/cage floor which might have given us a better idea of the possible sources of the PB and subsequent microbiome.

There are many questions that arise from our work that will be the subject of future research. However, this study lays the foundation of our knowledge of how the tonsillar microbiome develops in pigs in the first hours and weeks of age and how weaning affects

this microbiome. To the best of our knowledge, this is the first published study that follows the development of the tonsillar microbiome in any mammal during the first weeks of life.

CONCLUSIONS

Our data demonstrate a temporal succession in the development of the pig tonsillar microbiome through the first weeks of life. Many of the organisms found in the piglet oropharynx and tonsils immediately after birth disappear rapidly, within the first few hours of life, while other organisms acquired from the vaginal tract, such as *Streptococcaceae* and *Pasteurellaceae*, colonize and multiply. Additional organisms are also acquired rapidly from the sow teat skin, and possibly from milk, and eventually also from feces. The composition of the PB newborn piglet tonsil microbiome initially can be differentiated by litter and clusters mainly with the sow teat skin microbiome. Nevertheless, over the next three weeks, the composition and structure of the tonsil microbiome reaches a common point of development, showing a high degree of similarity among all piglets, regardless of litter and just prior to weaning. However, there was a dramatic change in the post weaning tonsillar microbiome, which was likely engendered by a combination of change in diet, change in environment, and addition of in- feed antibiotic, demonstrating the effect that weaning management practices exert in shaping tonsillar microbial communities. This research demonstrates the need for further studies to elucidate the role of antibiotic supplementation of feed in the development of tonsillar microbial communities, specifically when administered during the highly susceptible time of weaning.

APPENDIX

Table 2.2. Core microbiome at OTU level for litters and sow samples.

Sow parity	1	1	2	5	6	10				
OTUs	Litter 1700	Litter 1707	Litter 1631	Litter 1445	Litter 1402	Litter 1711	Sow Teats	Sow Vagina	Sow Tonsils	Sow Feces
Otu00001__Pasteurellaceae__	0.5	0.0	0.4	9.9	3.8	19.2	1.0	20.1	2.4	0.0
Otu00002__Streptococcaceae__Streptococcus	0.9	0.2	28.4	22.2	2.1	24.5	4.0	1.3	2.6	0.0
Otu00003__Moraxellaceae__Moraxella	0.5	0.0	24.2	11.7	0.6	14.4	3.9	0.3	0.9	0.0
Otu00004__Comamonadaceae__	2.3	0.0	0.0	0.0	0.3	0.0	1.3	1.1	0.4	0.0
Otu00005__Enterobacteriaceae__Escherichia/Shigella	0.3	0.0	0.0	0.2	14.7	0.1	3.2	7.4	0.2	0.6
Otu00007__Micrococcaceae__Rothia	1.1	2.4	23.0	5.2	0.9	10.5	4.6	0.1	0.2	0.0
Otu00009__Staphylococcaceae__Staphylococcus	6.1	4.8	3.1	14.8	0.7	25.6	2.8	0.3	0.2	0.0
Otu00010__Clostridiaceae_1__Clostridium_sensu_stricto	1.1	6.1	0.1	0.0	7.2	0.0	3.0	0.6	13.9	2.3
Otu00012__Streptococcaceae__Streptococcus	1.0	0.0	0.0	13.7	1.3	0.0	0.5	0.0	0.4	0.0
Otu00016__Clostridiaceae_1__Clostridium_sensu_stricto	1.2	1.2	0.6	0.9	3.6	0.0	3.9	13.3	2.4	0.4
Otu00017__Fusobacteriaceae__Fusobacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.1	0.0
Otu00018__Peptostreptococcaceae__Clostridium_XI	0.7	1.9	0.0	0.1	5.0	0.0	1.7	1.0	8.7	0.6
Otu00020__Erysipelotrichaceae__Turicibacter	0.6	2.8	0.1	0.0	5.4	0.0	1.9	0.2	8.7	0.5
Otu00021__Aerococcaceae__Aerococcus	0.7	0.3	0.0	0.0	3.5	0.0	0.7	14.3	0.0	0.0
Otu00023__Clostridiaceae_1__Clostridium_sensu_stricto	0.4	3.0	0.2	0.0	3.8	0.0	1.9	0.4	7.8	2.5
Otu00024__Bacillales_Incertae_Sedis_XI__Gemella	0.6	0.0	0.3	0.0	1.0	0.0	0.0	0.0	0.6	0.0
Otu00025__Peptostreptococcaceae__Clostridium_XI	0.3	3.6	0.0	0.0	3.8	0.0	1.0	0.1	7.0	0.4
Otu00027__Veillonellaceae__Veillonella	0.1	0.0	0.0	0.0	0.0	2.2	0.0	1.1	0.1	0.0
Otu00030__Clostridiaceae_1__Clostridium_sensu_stricto	0.2	2.3	0.1	0.1	2.2	0.0	0.9	0.1	4.0	0.8
Otu00034__Corynebacteriaceae__Corynebacterium	3.3	0.0	0.0	0.0	0.5	0.3	0.7	1.2	0.5	0.0
Otu00035__Pasteurellaceae__Pasteurella	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0
Otu00037__Clostridiales_Incertae_Sedis_XI__Tissierella	1.8	0.0	0.5	1.0	0.0	0.0	1.6	0.9	0.6	0.0
Otu00038__Micrococcaceae__Micrococcus	1.0	2.1	0.0	0.0	0.7	0.0	0.0	1.1	0.0	0.0
Otu00042__Ruminococcaceae__	0.9	3.4	1.2	0.4	0.1	0.0	0.7	0.1	0.4	5.2
Otu00044__Neisseriaceae__	0.0	0.0	0.0	0.1	0.0	0.2	0.0	0.1	0.8	0.0
Otu00045__Moraxellaceae__Acinetobacter	1.1	1.9	0.2	0.1	0.2	0.0	0.5	1.2	0.4	0.0
Otu00046__Comamonadaceae__	1.2	0.1	0.1	0.5	0.5	0.1	0.5	0.3	0.3	0.0
Otu00047__Streptococcaceae__Streptococcus	0.4	0.5	0.0	0.0	4.7	0.0	1.5	0.0	0.1	1.5
Otu00048__Lactobacillaceae__Lactobacillus	0.6	5.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.9
Otu00049__Moraxellaceae__Acinetobacter	0.8	0.0	0.1	0.1	0.2	0.2	0.4	0.4	0.5	0.0
Otu00051__Corynebacteriaceae__Corynebacterium	2.2	0.2	0.0	0.2	1.2	0.1	0.4	0.0	0.2	0.0
Otu00054__Clostridiales_Incertae_Sedis_XI__Sedimentibacter	3.9	0.0	0.0	0.0	0.0	0.1	0.6	0.0	0.6	0.0
Otu00056__Enterobacteriaceae__Yersinia	2.8	0.0	0.1	0.0	0.0	0.1	0.3	1.1	0.4	0.0
Otu00059__Lactobacillaceae__Lactobacillus	0.0	2.3	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.4
Otu00061__Comamonadaceae__	0.1	0.9	0.0	0.0	1.7	0.0	0.0	0.0	0.1	0.0
Otu00064__Aerococcaceae__	0.1	0.0	0.1	0.0	0.1	0.6	0.0	0.2	0.0	0.0
Otu00068__Bacillaceae_1__	0.6	0.0	0.3	0.3	0.0	0.0	0.9	0.1	0.4	0.0
Otu00070__Pasteurellaceae__Actinobacillus	0.0	0.0	0.0	0.0	0.7	0.2	0.8	0.5	1.4	0.0
Otu00073__Clostridiaceae_1__Clostridium_sensu_stricto	0.1	0.1	0.0	0.0	0.8	0.0	0.3	0.1	1.4	0.3
Otu00075__Enterobacteriaceae__	0.2	0.3	0.2	0.6	0.0	0.0	0.6	0.1	0.0	0.0
Otu00078__Ruminococcaceae__	0.0	0.8	0.2	0.0	0.2	0.1	0.5	0.1	0.2	1.4
Otu00080__Bifidobacteriaceae__Bifidobacterium	0.6	0.2	0.0	0.0	0.6	0.0	1.1	0.0	0.0	2.6
Otu00081__Sphingomonadaceae__Sphingomonas	0.8	0.0	0.1	0.3	0.0	0.0	0.1	0.1	0.4	0.0
Otu00082__Ruminococcaceae__Faecalibacterium	0.0	1.7	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0
Otu00084__Pseudomonadaceae__Pseudomonas	0.5	0.0	0.0	0.1	0.0	0.0	0.2	0.0	0.2	0.0
Otu00085__Streptococcaceae__Streptococcus	1.2	0.0	0.1	0.0	0.0	0.6	0.1	0.0	0.1	0.0
Otu00086__Lachnospiraceae__Clostridium_XlVb	0.0	0.0	0.3	0.4	0.0	0.0	0.4	0.3	0.1	0.0
Otu00087__Xanthomonadaceae__Ignatzschineria	1.8	0.0	0.4	0.3	0.0	0.0	0.0	0.1	0.0	0.0
Otu00089__Streptococcaceae__Streptococcus	1.0	0.0	0.0	0.0	0.8	0.0	0.1	0.0	0.1	0.0
Otu00091__Lachnospiraceae__Roseburia	0.0	0.5	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.3
Otu00095__Lactobacillaceae__Lactobacillus	0.1	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.0	1.1
Otu00096__Clostridiaceae_1__Clostridium_sensu_stricto	0.2	1.1	0.0	0.0	1.2	0.0	0.0	0.0	0.4	0.2
Otu00104__Erysipelotrichaceae__Sharpea	0.0	0.8	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.5
Otu00105__Ruminococcaceae__	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	1.1
Otu00108__Lachnospiraceae__	0.0	0.5	0.0	0.0	0.1	0.0	0.2	0.0	0.3	0.5
Otu00109__Prevotellaceae__	0.0	0.0	0.0	0.0	1.2	0.0	0.3	0.0	0.1	0.6
Otu00114__Veillonellaceae__Megasphaera	0.1	1.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	1.6
Otu00115__Ruminococcaceae__	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	2.0

Numbers in different colors identify core OTUs at different percentages of relative abundance. Red shows core OTUs at 1% relative abundance. Blue identify core OTUs at 0.1% relative abundance. Black color denotes OTUs that were not core.

Table 2.2. (cont´d).

Sow parity	1	1	2	5	6	10				
OTUs	Litter 1700	Litter 1707	Litter 1631	Litter 1445	Litter 1402	Litter 1711	Sow Teats	Sow Vagina	Sow Tonsils	Sow Feces
Otu00116__Erysipelotrichaceae__	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.1	0.0
Otu00117__Aerococcaceae__Aerococcus	0.1	1.6	0.0	0.0	0.0	0.0	0.3	0.2	0.1	0.0
Otu00118__Prevotellaceae__Paraprevotella	0.5	0.1	0.0	0.0	0.0	0.0	0.3	0.0	0.1	0.9
Otu00123__Nocardiaceae__Rhodococcus	2.0	0.0	0.0	0.0	0.0	0.0	0.2	0.3	0.1	0.0
Otu00124__Prevotellaceae__Prevotella	0.2	0.3	0.0	0.0	0.0	0.0	0.1	0.0	0.0	1.4
Otu00125__Corynebacteriaceae__Turicella	0.8	0.0	0.0	0.1	0.0	0.2	0.0	0.5	0.0	0.0
Otu00126__Corynebacteriaceae__Corynebacterium	0.9	0.2	0.0	0.0	0.0	0.0	0.2	0.3	0.1	0.0
Otu00130__Propionibacteriaceae__Propionibacterium	0.0	0.2	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0
Otu00135__Veillonellaceae__	0.3	0.0	0.0	0.0	0.3	0.0	0.2	0.0	0.1	0.3
Otu00151__Chitinophagaceae__	0.2	1.3	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
Otu00155__Erysipelotrichaceae__Bulleidia	0.8	0.3	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.5
Otu00158__Succinivibrionaceae__Succinivibrio	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.6
Otu00163__Spirochaetaceae__Treponema	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	2.3
Otu00172__Xanthomonadaceae__Dokdonella	1.5	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Otu00175__Acidaminococcaceae__	0.0	0.2	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.6
Otu00178__Veillonellaceae__Mitsuokella	0.0	0.5	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.1
Otu00185__Paenibacillaceae_1__	0.0	0.0	0.0	0.2	0.0	0.0	0.1	0.1	0.0	0.0
Otu00193__Porphyromonadaceae__	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2
Otu00204__Ruminococcaceae__	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.3
Otu00205__Lachnospiraceae__	0.0	0.4	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.4
Otu00211__Lachnospiraceae__Cellulosilyticum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0
Otu00217__Prevotellaceae__Prevotella	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8
Otu00219__Spirochaetaceae__	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.9
Otu00223__Actinomycetaceae__Trueperella	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.5	0.0	0.0
Otu00238__Moraxellaceae__Moraxella	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Otu00253__Ruminococcaceae__	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.2
Otu00260__Desulfovibrionaceae__Desulfovibrio	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.1	0.4
Otu00274__Ruminococcaceae__	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.2
Otu00282__Spirochaetaceae__Treponema	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2
Otu00307__Prevotellaceae__Prevotella	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2
Otu00318__Lachnospiraceae__	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2
Otu00320__Lachnospiraceae__Blautia	0.0	0.4	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.2
Otu00330__Ruminococcaceae__	0.3	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.3
Otu00340__Clostridiaceae_1__Clostridium_sensu_stricto	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2
Otu00347__Ruminococcaceae__	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.3
Otu00355__Lachnospiraceae__	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2
Otu00385__Ruminococcaceae__	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.3
Otu00392__Lachnospiraceae__Clostridium_XlVa	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0
Otu00426__Porphyromonadaceae__Dysgonomonas	0.0	0.0	0.1	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Otu00427__Prevotellaceae__Prevotella	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.5
Otu00467__Veillonellaceae__	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3
Otu00474__Geobacteraceae__Geobacter	0.0	0.0	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Otu00565__Porphyromonadaceae__	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4
Otu00583__Porphyromonadaceae__	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3
Otu00704__Ruminococcaceae__	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2
Otu00874__Spirochaetaceae__Treponema	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2

Numbers in different colors identify core OTUs at different percentages of relative abundance. Red shows core OTUs at 1% relative abundance. Blue identify core OTUs at 0.1% relative abundance. Black color denotes OTUs that were not core.

Table 2.3. SIMPER analysis between litters through different sampling times

Litter	Sampling time	n.i.	Y1700	Y1707	Y1631	Y1445	Y1402	Y1711
Y1700	Newborn	4		87.73	92.22	87.89	86.88	89.15
parity=1	First week	4		60.12	46.65	51.89	49.32	57.05
	Second week	4			40.05	42.06	94.42	35.88
	Third week	4		33.43	26.6	27.04	33	26.31
	Fourth week	4			36.71	47.98	35.21	42.54
Y1707	Newborn	3	87.73		94.04	92.37	78.23	92.5
parity=1	First week	4	60.12		56.12	40.06	51.07	34.75
	Second week	0						
	Third week	1	33.43		36.51	35.25	23.5	32.55
	Fourth week	0						
Y1631	Newborn	4	92.22	94.04		56.62	93.91	52.16
parity=2	First week	4	46.65	56.12		46.95	44.42	55.05
	Second week	4	40.05			44.01	96.42	42.58
	Third week	4	26.6	36.51		30.04	33.48	30.27
	Fourth week	4	36.71			47.21	32.04	41.19
Y1445	Newborn	4	87.89	92.37	56.62		89.14	45.82
parity=5	First week	4	51.89	40.06	46.95		46.22	39.17
	Second week	4	42.06		44.01		95.79	38.2
	Third week	4	27.04	35.25	30.04		37.32	26.03
	Fourth week	4	47.98		47.21		45.09	42.49
Y1402	Newborn	3	86.88	78.23	93.91	89.14		91.8
parity=6	First week	4	49.32	51.07	44.42	46.22		54.72
	Second week	1	94.42		96.42	95.79		96.63
	Third week	1	33	23.5	33.48	37.32		35.61
	Fourth week	1	35.21		32.04	45.09		37.48
Y1711	Newborn	4	89.15	92.5	52.16	45.82	91.8	
parity=10	First week	4	57.05	34.75	55.05	39.17	54.72	
	Second week	4	35.88		42.58	38.2	96.63	
	Third week	4	26.31	32.55	30.27	26.03	35.61	
	Fourth week	4	42.54		41.19	42.49	37.48	

Data displayed represent values of overall average dissimilarity, when a comparison between litter was performed for the different sampling times.
n.i.: Number of samples included in the analysis
parity: Total number of deliveries that the sow had when was sampled

Table 2.4. Core microbiome at OTU level for third week

OTUs	Litter 1700	*Litter 1707	Litter 1631	Litter 1445	*Litter 1402	Litter 1711	Average
Otu00001__Pasteurellaceae__	23.1	16.5	23.3	22.3	18.5	23.7	21.2
Otu00002__Streptococcaceae__Streptococcus	5.6	10.3	6.2	7.3	5.6	8.6	7.3
Otu00003__Moraxellaceae__Moraxella	27.6	40.7	24.1	26.3	38.9	28.4	31.0
Otu00006__Porphyromonadaceae__	4.6	2.6	7.2	5.9	2.8	4.2	4.5
Otu00007__Micrococcaceae__Rothia	0.4	0.5	0.5	0.6	0.7	0.1	0.4
Otu00008__Moraxellaceae__	5.7	3.8	10.1	0.2	5.5	1.8	4.5
Otu00010__Clostridiaceae_1__Clostridium_sensu_stricto	0.1	0.6	0.5	0.4	3.6	0.2	0.9
Otu00012__Streptococcaceae__Streptococcus	0.3	0.6	2.0	0.2	1.1	0.4	0.8
Otu00015__Prevotellaceae__	0.6	3.2	1.3	1.9	0.7	1.9	1.6
Otu00016__Clostridiaceae_1__Clostridium_sensu_stricto	0.0	0.2	0.1	0.1	0.2	0.1	0.1
Otu00017__Fusobacteriaceae__Fusobacterium	4.0	1.1	3.0	5.3	1.4	4.4	3.2
Otu00018__Peptostreptococcaceae__Clostridium_XI	0.2	0.1	0.6	0.3	1.6	0.1	0.5
Otu00020__Erysipelotrichaceae__Turicibacter	0.1	0.1	0.3	0.1	1.0	0.1	0.3
Otu00023__Clostridiaceae_1__Clostridium_sensu_stricto	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Otu00024__Bacillales_Incertae_Sedis_XI__Gemella	2.1	1.0	0.8	0.8	0.8	0.6	1.0
Otu00025__Peptostreptococcaceae__Clostridium_XI	0.1	0.0	0.3	0.2	2.0	0.0	0.4
Otu00026__Flavobacteriaceae__	2.3	2.3	2.7	5.1	1.2	3.8	2.9
Otu00027__Veillonellaceae__Veillonella	0.1	0.2	0.2	0.4	0.0	0.1	0.2
Otu00028__Leptotrichiaceae__	3.2	0.3	3.2	1.9	0.1	3.9	2.1
Otu00030__Clostridiaceae_1__Clostridium_sensu_stricto	0.0	0.0	0.2	0.0	0.8	0.0	0.2
Otu00035__Pasteurellaceae__Pasteurella	0.1	0.0	0.1	0.1	0.3	0.1	0.1
Otu00041__Neisseriaceae__Neisseria	0.2	0.0	0.6	0.2	0.4	0.2	0.2
Otu00042__Ruminococcaceae__	0.0	0.8	0.1	0.1	0.3	0.0	0.2
Otu00043__Flavobacteriaceae__	0.6	0.0	0.3	0.8	0.4	0.9	0.5
Otu00044__Neisseriaceae__	0.2	0.0	0.4	2.8	0.0	0.1	0.6
Otu00045__Moraxellaceae__Acinetobacter	0.0	0.1	0.1	0.0	0.0	0.0	0.0
Otu00048__Lactobacillaceae__Lactobacillus	0.0	0.0	0.0	0.1	0.1	0.0	0.0
Otu00050__Bacteroidaceae__Bacteroides	0.3	0.0	0.4	0.5	0.0	1.0	0.4
Otu00051__Corynebacteriaceae__Corynebacterium	0.0	0.3	0.0	0.0	0.0	0.0	0.1
Otu00064__Aerococcaceae__	0.0	0.0	0.1	0.2	0.3	0.0	0.1
Otu00070__Pasteurellaceae__Actinobacillus	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Otu00071__Prevotellaceae__Prevotella	0.1	0.4	0.4	0.7	0.0	0.0	0.3
Otu00072__Ruminococcaceae__	2.5	2.0	0.0	1.0	0.3	0.0	0.9
Otu00076__Lachnospiraceae__	0.2	0.0	0.2	0.3	0.0	0.5	0.2
Otu00077__Carnobacteriaceae__	0.2	0.6	0.3	0.1	0.8	0.4	0.4
Otu00078__Ruminococcaceae__	0.0	0.0	0.1	0.1	0.1	0.0	0.1
Otu00079__Bacteroidaceae__Bacteroides	0.4	0.0	0.5	0.8	0.2	1.3	0.5
Otu00085__Streptococcaceae__Streptococcus	0.1	0.3	0.0	0.1	0.1	0.0	0.1
Otu00091__Lachnospiraceae__Roseburia	0.0	0.0	0.0	0.0	0.5	0.0	0.1
Otu00094__Streptococcaceae__Lactococcus	0.0	0.3	0.0	0.0	0.0	0.0	0.1
Otu00097__Alcaligenaceae__	0.2	0.3	0.3	0.3	0.0	0.0	0.2
Otu00098__Clostridiales_Incertae_Sedis_XI__Helcococcus	0.1	0.0	0.2	0.6	0.0	0.7	0.3
Otu00108__Lachnospiraceae__	0.1	0.0	0.1	0.1	0.3	0.0	0.1
Otu00122__Leptotrichiaceae__Leptotrichia	0.0	0.3	0.0	0.1	0.0	0.6	0.2
Otu00127__Fusobacteriaceae__Fusobacterium	0.2	0.0	0.1	0.2	0.0	0.1	0.1
Otu00130__Propionibacteriaceae__Propionibacterium	0.0	1.9	0.0	0.0	0.1	0.0	0.3
Otu00143__Campylobacteraceae__Campylobacter	0.1	0.0	0.0	0.4	0.0	0.3	0.1
Otu00147__Erysipelotrichaceae__	0.1	0.2	0.0	0.1	0.0	0.2	0.1
Otu00149__Bacteria_unclassified__	0.0	0.0	0.1	0.1	0.2	0.0	0.0
Otu00156__Leptotrichiaceae__Sneathia	0.6	0.0	0.0	0.0	0.0	0.0	0.1
Otu00159__Alcaligenaceae__	0.0	0.3	0.0	0.0	0.0	0.0	0.1
Otu00162__Succinivibrionaceae__Succinivibrio	0.0	0.0	0.0	0.0	0.4	0.0	0.1
Otu00174__Erysipelotrichaceae__	0.0	0.2	0.0	0.0	0.0	0.0	0.0
Otu00176__Dermabacteraceae__Brachybacterium	0.0	1.1	0.0	0.0	0.0	0.0	0.2
Otu00183__Actinomycetaceae__Actinomyces	0.0	0.0	0.0	0.2	0.0	0.2	0.1
Otu00195__Spirochaetaceae__	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Otu00200__Moraxellaceae__Moraxella	0.3	0.0	0.4	0.2	0.0	0.2	0.2
Otu00204__Ruminococcaceae__	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Otu00205__Lachnospiraceae__	0.0	0.0	0.0	0.1	0.1	0.0	0.0
Otu00209__Aerococcaceae__Facklamia	0.0	0.0	0.0	0.0	0.3	0.0	0.0
Otu00225__Leptotrichiaceae__	0.1	0.0	0.5	0.0	0.0	0.1	0.1
Otu00232__Fusobacteriaceae__Fusobacterium	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Otu00280__Spirochaetaceae__	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Otu00288__Dietziaceae__Dietzia	0.0	0.1	0.0	0.0	0.0	0.0	0.0
Otu00292__Bacteroidaceae__Bacteroides	0.0	2.1	0.0	0.0	0.0	0.0	0.3
Otu00311__Xanthomonadaceae__Stenotrophomonas	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Otu00324__Lachnospiraceae__	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Otu00338__Lachnospiraceae__	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Otu00346__Leptotrichiaceae__	0.0	0.0	0.1	0.0	0.1	0.1	0.0
Otu00347__Ruminococcaceae__	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Otu00510__Lachnospiraceae__	0.0	0.0	0.1	0.1	0.3	0.0	0.1
Otu00545__Cardiobacteriaceae__Suttonella	0.1	0.0	0.0	0.1	0.0	0.0	0.0
Otu00582__Lachnospiraceae__Clostridium_XlVa	0.0	0.0	0.0	0.0	0.3	0.0	0.1
Otu00653__Ruminococcaceae__	0.0	0.0	0.0	0.0	0.4	0.0	0.1
Otu00982__Clostridiales_Incertae_Sedis_XI__Anaerococcus	0.0	0.0	0.0	0.0	0.3	0.0	0.1
Otu01018__Oligosphaeraceae__Oligosphaera	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Otu01058__Ruminococcaceae__Butyricicoccus	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Otu01160__Ruminococcaceae__Faecalibacterium	0.0	0.0	0.0	0.0	0.4	0.0	0.1
Otu01326__Alcanivoracaceae__Alcanivorax	0.0	0.0	0.0	0.0	0.3	0.0	0.0
Otu01828__Lachnospiraceae__Clostridium_XlVa	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Otu02516__Ruminococcaceae__	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Otu03039__Moraxellaceae__Moraxella	0.0	0.1	0.0	0.0	0.0	0.0	0.0

Numbers in different colors identify core OTUs at different percentages of relative abundance. Red shows core OTUs at 1% relative abundance. Blue identify core OTUs at 0.1% relative abundance. Black color denotes OTUs that were not core. * indicates litters with less than 4 samples for this specific time.

Table 2.5. Core microbiome at OTU level for fourth week

OTUs	Litter 1700	Litter 1631	Litter 1445	*Litter 1402	Litter 1711	Average
Otu00001__Pasteurellaceae__	28.8	23.1	21.3	15.2	20.3	21.7
Otu00002__Streptococcaceae__Streptococcus	46.3	51.0	28.8	63.2	40.0	45.9
Otu00003__Moraxellaceae__Moraxella	3.0	0.9	9.0	5.1	13.1	6.2
Otu00005__Enterobacteriaceae__Escherichia/Shigella	0.5	0.2	0.3	0.0	0.1	0.2
Otu00007__Micrococcaceae__Rothia	1.2	0.7	2.5	4.3	1.3	2.0
Otu00008__Moraxellaceae__	0.1	0.0	2.9	1.1	2.9	1.4
Otu00010__Clostridiaceae_1__Clostridium_sensu_stricto	0.1	0.4	0.7	0.6	0.3	0.4
Otu00012__Streptococcaceae__Streptococcus	1.9	2.5	0.5	0.0	0.6	1.1
Otu00018__Peptostreptococcaceae__Clostridium_XI	0.0	0.0	0.2	0.0	0.0	0.1
Otu00019__Sphingomonadaceae__Sphingomonas	0.0	0.3	0.1	0.0	0.0	0.1
Otu00024__Bacillales_Incertae_Sedis_XI__Gemella	0.3	0.1	1.0	1.4	0.3	0.6
Otu00026__Flavobacteriaceae__	0.1	0.0	0.1	0.0	0.3	0.1
Otu00027__Veillonellaceae__Veillonella	1.1	0.7	1.5	0.0	0.4	0.7
Otu00034__Corynebacteriaceae__Corynebacterium	0.3	0.0	0.0	0.0	0.2	0.1
Otu00035__Pasteurellaceae__Pasteurella	0.0	0.1	0.2	0.0	0.0	0.1
Otu00037__Clostridiales_Incertae_Sedis_XI__Tissierella	0.1	0.2	0.2	0.0	0.2	0.1
Otu00041__Neisseriaceae__Neisseria	0.3	0.2	3.9	0.0	1.0	1.1
Otu00042__Ruminococcaceae__	0.0	0.1	0.2	0.0	0.1	0.1
Otu00044__Neisseriaceae__	0.0	0.2	1.9	0.0	0.8	0.6
Otu00045__Moraxellaceae__Acinetobacter	0.2	0.2	0.0	0.7	0.2	0.2
Otu00047__Streptococcaceae__Streptococcus	0.1	0.1	0.0	0.0	0.2	0.1
Otu00051__Corynebacteriaceae__Corynebacterium	0.0	0.1	0.0	0.4	0.1	0.1
Otu00052__Lactobacillaceae__Lactobacillus	4.8	0.6	1.2	0.0	0.4	1.4
Otu00054__Clostridiales_Incertae_Sedis_XI__Sedimentibacter	0.3	0.0	0.0	0.0	0.5	0.2
Otu00056__Enterobacteriaceae__Yersinia	0.1	0.0	0.0	0.0	0.2	0.1
Otu00059__Lactobacillaceae__Lactobacillus	0.8	0.1	0.4	0.0	0.3	0.3
Otu00064__Aerococcaceae__	0.1	0.0	0.1	0.0	0.2	0.1
Otu00066__Planococcaceae__	0.1	0.0	0.0	0.0	0.2	0.1
Otu00070__Pasteurellaceae__Actinobacillus	0.3	0.1	0.0	0.1	0.2	0.1
Otu00082__Ruminococcaceae__Faecalibacterium	0.3	0.6	0.6	0.5	0.5	0.5
Otu00091__Lachnospiraceae__Roseburia	0.2	0.5	0.4	0.8	0.7	0.5
Otu00097__Alcaligenaceae__	0.1	0.0	0.1	0.0	0.3	0.1
Otu00100__Lachnospiraceae__Blautia	0.2	0.5	0.9	0.7	0.6	0.6
Otu00104__Erysipelotrichaceae__Sharpea	0.2	0.1	0.9	0.2	0.3	0.3
Otu00105__Ruminococcaceae__	0.0	0.1	0.2	0.0	0.1	0.1
Otu00109__Prevotellaceae__	0.1	0.2	0.2	0.0	0.3	0.2
Otu00114__Veillonellaceae__Megasphaera	0.1	0.1	0.3	0.0	0.1	0.1
Otu00118__Prevotellaceae__Paraprevotella	0.0	0.0	0.2	0.0	0.0	0.0
Otu00124__Prevotellaceae__Prevotella	0.2	0.3	0.6	0.1	0.1	0.3
Otu00125__Corynebacteriaceae__Turicella	0.1	0.0	0.0	0.0	0.0	0.0
Otu00130__Propionibacteriaceae__Propionibacterium	0.0	0.0	0.0	0.1	0.0	0.0
Otu00135__Veillonellaceae__	0.0	0.1	0.4	0.0	0.2	0.2
Otu00140__Planococcaceae__	0.0	0.1	0.0	0.0	0.0	0.0
Otu00174__Erysipelotrichaceae__	0.0	0.0	0.1	0.9	0.0	0.2
Otu00181__Enterococcaceae__Enterococcus	0.1	0.2	0.2	0.0	0.1	0.1
Otu00193__Porphyromonadaceae__	0.0	0.1	0.2	0.0	0.2	0.1
Otu00194__Prevotellaceae__Prevotella	0.1	0.1	0.5	0.2	0.1	0.2
Otu00200__Moraxellaceae__Moraxella	0.0	0.0	0.0	0.1	0.0	0.0
Otu00240__Acidaminococcaceae__Acidaminococcus	0.0	0.0	0.3	0.0	0.1	0.1
Otu00261__Ruminococcaceae__Butyricicoccus	0.1	0.1	0.2	0.0	0.1	0.1
Otu00262__Prevotellaceae__Prevotella	0.0	0.0	0.2	0.0	0.0	0.1
Otu00269__Ruminococcaceae__	0.0	0.0	0.0	0.6	0.0	0.1
Otu00284__Veillonellaceae__Mitsuokella	0.0	0.0	0.5	0.0	0.0	0.1
Otu00307__Prevotellaceae__Prevotella	0.1	0.1	0.1	0.0	0.1	0.1
Otu00313__Lachnospiraceae__Dorea	0.1	0.2	0.1	0.0	0.1	0.1
Otu00315__Prevotellaceae__Alloprevotella	0.0	0.3	0.0	0.0	0.2	0.1
Otu00338__Lachnospiraceae__	0.0	0.1	0.1	0.3	0.1	0.1
Otu00352__Sutterellaceae__Sutterella	0.0	0.0	0.0	1.0	0.0	0.2
Otu00367__Ruminococcaceae__	0.1	0.1	0.1	0.0	0.1	0.1
Otu00438__Streptococcaceae__Streptococcus	0.2	0.1	0.1	0.1	0.1	0.1
Otu00480__Prevotellaceae__Alloprevotella	0.0	0.2	0.1	0.0	0.0	0.1
Otu00517__Lachnospiraceae__Blautia	0.0	0.1	0.1	0.0	0.0	0.0
Otu00519__Prevotellaceae__Prevotella	0.0	0.1	0.2	0.0	0.1	0.1
Otu00543__Ruminococcaceae__Ruminococcus	0.0	0.1	0.1	0.0	0.0	0.0
Otu00609__Lachnospiraceae__Ruminococcus2	0.0	0.2	0.1	0.0	0.0	0.1
Otu00637__Ruminococcaceae__	0.0	0.0	0.0	0.4	0.1	0.1
Otu00660__Erysipelotrichaceae__	0.0	0.0	0.3	0.0	0.0	0.1
Otu00675__Erysipelotrichaceae__	0.0	0.0	0.0	0.1	0.0	0.0
Otu00694__Streptococcaceae__Streptococcus	0.0	0.0	0.0	0.3	0.0	0.1
Otu00783__Streptococcaceae__Streptococcus	0.0	0.0	0.1	0.2	0.0	0.1
Otu01058__Ruminococcaceae__Butyricicoccus	0.0	0.0	0.1	0.0	0.0	0.0
Otu01339__Alcaligenaceae__Oligella	0.0	0.0	0.0	0.3	0.0	0.1

Numbers in different colors identify core OTUs at different percentages of relative abundance. Red shows core OTUs at 1% relative abundance. Blue identify core OTUs at 0.1% relative abundance. Black color denotes OTUs that were not core. * indicates litters with less than 4 samples for this specific time. The absence of litter 1707 samples means that there were not available samples in this time.

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CHAPTER 3. DEVELOPMENT OF THE TONSILLAR MICROBIOME IN PIGS FROM NEWBORN TO MARKET AGE

INTRODUCTION

Numerous bacteria and viruses can access the host using the oropharynx and nasopharynx as portals of entrance. Tonsils, lympho-epithelial tissues located at their junction, play a key role in surveillance of pathogens accessing host [1]. Tonsils play a significant role in the initial process of pathogen-host colonization and as a reservoir of host-specific pathogens as well as zoonotic pathogens highly transmissible to humans [2]. Multiple bacterial pathogens are regularly isolated from tonsils of asymptomatic animals. Pathogens residing in the tonsils can spread systemically or be transmitted to other animals including humans, with such transmission often triggered by stressful conditions such as transport [3]. It has been suggested that in the process of the colonization of host by pathogenic microorganisms, the microbiome plays an important preventive role [4-7] as well as a regulatory role in resistance to infection [8, 9].

There are limited numbers of studies addressing the tonsillar microbiome in humans or pigs [10-16]. Conversely, there is a growing number of studies on intestinal microbiome in different species. Some studies suggest a gradual and sequential process in the development of intestinal microbiome [17, 18], where some taxa persisted and were stable while others were acquired over time. However, microbial communities tended to achieve an adult-like profile as time progressed [17, 19]. This trend was seen despite the fact that during development there were significant shifts in the structure of the population [19] as well as in the diversity [18], and many of these shifts were associated with life events, for example diet changes and antibiotic treatment, among others [17].

It has been demonstrated that common management practices such as the use of antibiotic treatments can significantly affect microbial communities and predispose the host to infections [20]. However, the microbiota also can be shifted towards a microbial community that would protect the host from potential infections, as in the case of altering the intestinal microbiota through fecal microbiota transplantation [6]. Notwithstanding the relevant role that the microbiota can play in maintaining a good health status in the host, very little is known about the tonsillar microbial communities of pigs based on a culture-independent approach. Two studies have described the normal tonsillar microbiome in finishing pigs [12, 15] and one study has described the metabolically active microbiome of slaughter pigs [10]. The core tonsil microbiome in 18-20 week old grower-finisher pigs was comprised of members of the families *Pasteurellaceae, Moraxellaceae, Streptococcaceae, Fusobacteriaceae*, *Veillonellaceae, Enterobacteriaceae, Neisseriaceae,* and *Peptostreptococcaceae,* as well as the order *Clostridiales*. Currently, tonsils are an under-explored habitat of the mammalian microbiome. How the microbial community in the tonsils is established and develops over time and what role it plays in the acquisition and carriage of pathogens and thus in host health and disease is not known at this time. Microbiome development is suggested to be based on specific bacterial interactions and not on random assembly of microorganisms [17]. Numerous authors have shown that human or animal microbiome development in different body locations frequently follows a gradual and successional process [17-19, 21]. Whether a similar process occurs in tonsils remains to be established.

The goal of the current study was to utilize a culture-independent approach using high-throughput sequencing of 16S rRNA genes to follow and describe the development of the tonsillar microbial communities in pigs from birth through market age. This characterization of the development of the swine tonsillar microbiome lays a base for future studies to alter this microbiome to reduce pathogen load and improve overall animal health.

MATERIALS AND METHODS

Animals. The Michigan State University Institutional Animal Care and Use Committee approved this study and the animal procedures. The pigs used in this study were from a high health status farrow-to-finish herd with ~200 sows. Relevant medical history for this herd included no recent respiratory disease; freedom from *Actinobacillus pleuropneumoniae, Mycoplasma hyopneumoniae*, and PRRSV; a recent outbreak of porcine epidemic diarrhea virus (PEDV), under control prior to this study; and routine vaccination against erysipelas, atrophic rhinitis, and porcine circovirus type 2 (PCV2).

Four crossbred sows (Yorkshire X Hampshire) of different parity (number of pregnancies) were selected for this study and included sow 1700 (first parity), sow 1631 (second parity), sow 1445 (fifth parity) and sow 1711 (tenth parity). Four randomly selected piglets from each of the four sows were sampled within a period no longer than 8 hours after birth (newborn) and the same piglets were sampled subsequently at 1, 2, 3, 4, 6, 8, 10, 12, 16, and 19 weeks of age.

Newborn piglets received a single intramuscular injection of Iron-Dextran during their first week of life. Between the third and fourth weeks of age (21 to 24 days – average weight 18 pounds) piglets were weaned, vaccinated, and moved from the farrowing room where they were housed with the sow and littermates to a nursery room, with litters maintained as pen mates. At this time the piglets were weaned from milk to a solid pellet ration diet (Pig 1300®, Akey Nutrition, Brookville OH) supplemented with Carbadox® at a dose of 50 g/ton. Two weeks after being moved to the nursery facility (at 5 weeks of age), Carbadox® supplementation was removed from the feed, and food was changed to a ground ration supplemented with Tylan® at a dose of 100 g/ton. At ~nine weeks of age (63 to 67 days – average weight 60 pounds), piglets were moved to a finishing room and were assigned to different pens based on criteria such as gender and weight; separation by litter was no longer maintained. At this time, Tylan® supplementation was discontinued and a ground ration without supplementation was provided. Finally, at ~eighteen weeks of age, piglets were moved again to another finishing room (with mixing of prior penmates) where they remained until being moved to the slaughterhouse (average weight 240 pounds). These management practices are summarized in Figure 3.1.

Collection of microbiome samples. Tonsil brushes developed by our group and validated in previous studies [12] were used to collect tonsil microbiome samples from sows and larger piglets, while Cytosoft™ cytology brushes (Medical Packaging Corporation, Camarillo, CA) were used for smaller piglets. Collection and storage of samples was as previously described [12].

Isolation of community DNA. Extraction of community DNA from samples was performed using a PowerSoil DNA Isolation Kit and PowerBead tubes (MoBio Laboratories, Carlsbad, CA) as previously described [12, 22].

Illumina sequencing and sequence analysis. Sequencing was performed at the MSU Research Technology Support Facility (RTSF) as previously described [22]. Negative controls consisting of DNA-free water or MoBio C6 reagent were used as "blank library controls" [23] and included in each sequencing run. Briefly, uniquely indexed primers were used to amplify the V4 region of the 16S rRNA gene from the community DNA, as described by Caporaso [24]. A SequalPrep normalization plate (Invitrogen) was used to normalize the amplification products, which were then pooled and the reaction cleaned using AMPure XP beads. The pooled sample was sequenced on an Illumina MiSeq v2 flow cell using a 500 cycle v2 reagent kit (PE250 reads). Base calling was performed using Illumina Real Time Analysis Software (RTA) v1.18.54 and output of RTA demultiplexed and converted to FastQ files using Illumina Bcl2fastq v1.8.4.

The open-source, platform-independent, community-supported software program mothur v.1.35.0 (http://www.mothur.org) [25] was used for amplicon analysis. Raw sequencing data was processed according to the mothur standard operating procedure (http://www.mothur.org/wiki/MiSeq_SOP) [26] and aligned using the mothur-formatted version 123 of Silva 16S ribosomal gene database [27]. After sequences were classified, all sequences classified as Chloroplast, Mitochondria, unknown, Archaea, or Eukaryota were removed from the data set. Subsampling at 7000 sequences per sample was done, followed by a preclustering of the sequences and

removal of chimeric sequences using a mothur formatted version of the Ribosomal Database Project (RDP) training set version 14 and uchime, based on mothur protocol. A cutoff of ≥97% sequence identity was used to classify sequences into Operational Taxonomic Units (OTUs). Singleton and doubleton reads were removed before the final analysis. For the final analysis of the data, samples were subsampled to 5179 reads per sample. The full data set analyzed is available as a supplemental file at (https://figshare.com/s/e910143b694abb664fcc). Samples from newborn piglets through weaning were used in a smaller study [22]

Diversity and statistical analysis. A clustering cutoff of 3% for the processed sequences was used in the statistical analysis. Mothur output files were used to estimate alpha diversity (sobs) and beta diversity indexes, as well as representative sequences, all of which were calculated in mothur v.1.35.0 (http://www.mothur.org) [25]. PAST3 (Version 3.14; http://folk.uio.no/ohammer/past/) was used for statistical analysis of the samples. FigTree (Version 1.4.3; http://tree.bio.ed.ac.uk/software/figtree/) was used for construction of dendrogram figures. ImageJ was used to measure the area of the ellipses for the two dimensional scatter plot [28]. RStudio (Version 0.99.446; https://www.rstudio.com/) and library gplots (https://CRAN.R-project.org/package=gplots) were used to generate heatmaps. Inkscape 0.91 (https://inkscape.org/en/download/mac-os/), was used to process images and edit labels. Taxonomy tables and OTU plots were generated in Microsoft® Excel® 2011, where the analysis of samples was done with data that represented higher than 0.1% of the total reads for the samples analyzed.

Availability of supporting data. Raw sequence data and metadata is available at NCBI database (SRA accession number: in submission process).

RESULTS

A total of 128 tonsil microbiome samples from pigs from birth through market age were sequenced and analyzed. Of these, 64 samples were collected from piglets before weaning (newborn – third week), which included samples from 16 piglets (4 per sow) at each time point. In addition, 64 samples were collected from pigs after weaning, which included samples from 16 piglets at week 4 and 8 piglets (2 per sow) at all subsequent time points (Table 3.1.).

Table 3.1. Samples processed by sampling time and litter

	Sow ID	1700	1631	1445	1711
	# sow parity	1	2	5	10
	Litter members	10,11,12,13	15,16,17,18	36,39,40,42	22,23,24,26
ID	Sampling time	number of samples analyzed per sampling time			
A	Newborns	4	4	4	4
B	First week	4	4	4	4
C	Second week	4	4	4	4
D	Third week	4	4	4	4
	Total samples before weaning	16	16	16	16
E	Fourth week	4	4	4	4
F	Sixth week	2	2	2	2
G	Eighth week	2	2	2	2
H	Tenth week	2	2	2	2
I	Twelfth week	2	2	2	2
J	Sixteenth week	2	2	2	2
K	Nineteenth week	2	2	2	2
	Total samples after weaning	16	16	16	16

Management practices are related with changes in population diversity. Tonsil samples for microbiome analysis were collected at eleven time points during the life of the pigs in this study. Some of the sampling times were chosen specifically to represent times associated with management practices significant in the life of the pigs, including immediately prior to and after weaning, alteration in feed and in-feed growth promoters, and movement to new rooms (Figure 3.1.)

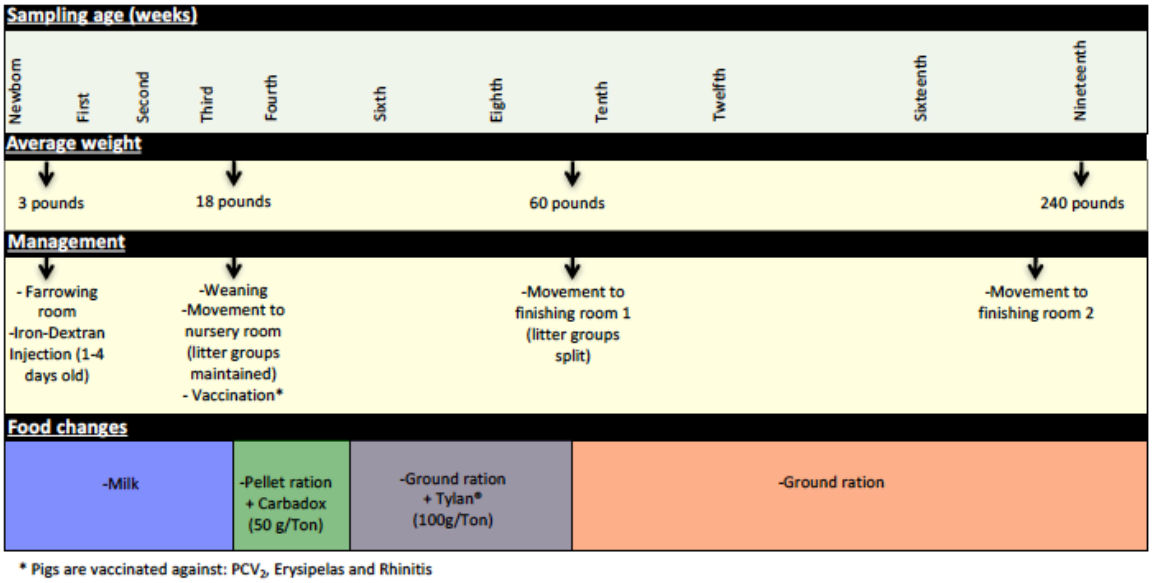

Figure 3.1. Significant management practices at the swine farm during the life of the pigs in this study. General management features experienced by the pigs during their life at the farm are depicted here, including changes in feed, use of in feed antibiotics, and movement to new housing.

Analysis of the alpha diversity of the tonsil microbiome (sobs: refers to the number of observed OTUs) and the relation with the different changes experienced by the pigs during their life showed that the alpha diversity varied widely (Table 3.2). For newborn piglets, the average value of sobs was 110 (range = 26-376, SD = 101). The average sobs value decreased steadily in the following weeks (first to third week),

reaching a value of 83 (range = 57-132). This was accompanied by a marked decrease in the standard deviation to 24, indicating that the microbiome became very similar in all pigs by 3 weeks of age. In contrast, from week four to ten there was an abundant increase in diversity that coincided with specific challenging events experienced by the piglets. Between the third and fourth week, the piglets were weaned, and at the same time they were moved to a nursery room, vaccinated and their diet was changed. These changes were reflected in a slight increase in the average and maximum sobs as well as the standard deviation. However, the biggest change in the diversity occurred during the period where Carbadox® was removed from the diet and Tylan® was supplemented. Diversity increased to over three times the previous registered values for average sobs. Conversely, the removal of Tylan®, accompanied by the transfer of pigs to a finishing room where they were no longer segregated by litter, led to trend of decreasing diversity. By week nineteen, this progressive decrease in the diversity led to a value of average sobs of 108 (range = 73-178, SD = 35). Overall, there was a pattern demonstrating that extended time under constant conditions led to fewer sobs and reduced standard deviation.

Table 3.2. Number of observed OTUs (sobs) during the different sampling times

Sampling time	# Samples analyzed	Average sobs	Min sobs	Max sobs	Standard Deviation sobs
Newborn	16	110	26	376	100.6
First week	16	104	37	223	58.1
Second week	16	107	49	242	59.0
Third week	16	83	57	132	23.8
	Weaning, Carbadox supplementation, Moved to nursery, Vaccinated				
Fourth week	16	111	53	175	38.7
	Carbadox removal, Supplementation with Tylan				
Sixth week	8	368	215	434	69.8
Eighth week	8	355	276	408	46.5
	Tylan removal, Moved to finisher room 1, Litters split				
Tenth week	8	257	123	377	96.6
Twelfth week	8	139	53	234	64.1
Sixteenth week	8	132	83	177	35.7
	Moved to finisher room 2				
Nineteenth week	8	108	73	178	35.2

sobs: number of observed OTUs
Min sobs: Minimum number of observed sobs
Max sobs: Maximum number of observed sobs

Challenging management conditions during development of the pigs generated disruption in the microbiome. We wondered if the development of the tonsillar microbiome followed a succession in time and if there was any similarity in the microbiome between the different sampling periods. An unrooted dendrogram based on a Bray-Curtis analysis (Figure 3.2.) shows the clustering of the pig tonsillar microbiome samples from newborn through the nineteenth week. Samples from newborn piglets were mainly distributed in two groups, one corresponding to pigs from a first parity sow (4/16; 25%) and the other from pigs of multiparous sows (10/16; 62.5%), the remaining two samples were clustered with microbiome samples of older pigs. At one week of age, the microbiome samples were clustered by litter in four different groups.

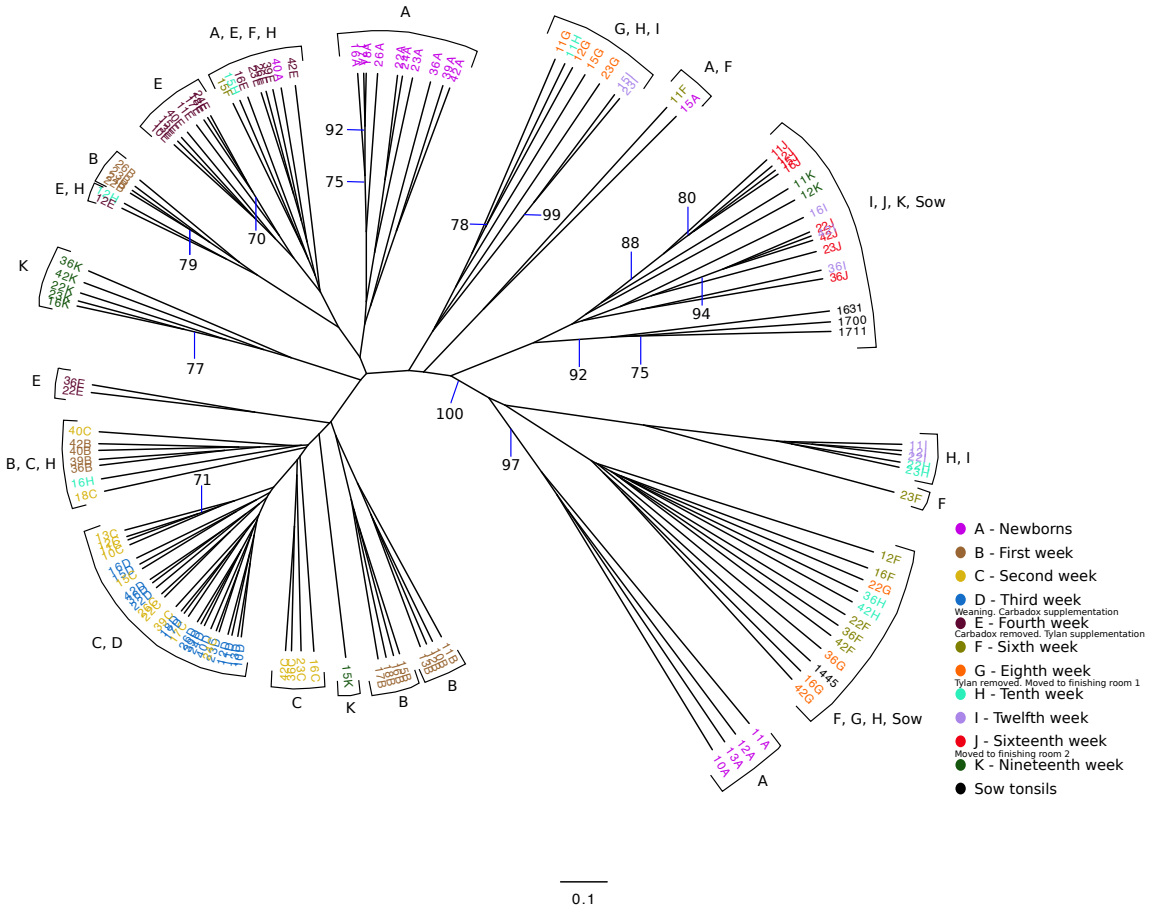

Figure 3.2. Unrooted Bray-Curtis dendrogram for all sampled weeks. The dendrogram shows the clustering of the samples collected from pigs from newborn through nineteen weeks, as well as sow tonsillar samples. Samples are color coded by week of sampling. Small legends indicate some of the challenges that took place in specific times.

The following weeks showed that as pigs aged, their tonsil microbiomes tended to become more similar. During the second week, the samples clustered together in three related groups. In the third week, all sixteen samples clustered together in one group, which also included ten of the week 2 samples. The fourth week, which marked a transitional time after a challenge, i.e., weaning plus movement to new housing plus addition of Carbadox® to the new solid feed, showed a split of the previously tightly clustered samples into four separate groups, which were not clustered by litter. The sixth week, again marked by a transition after a challenge, i.e., removal of Carbadox® and addition of Tylan® to the feed, again showed samples clustered in four separate groups, which were neither clustered by litter nor the same, with one exception, as the groups for the fourth week samples. However, for the eighth week, samples clustered in only two groups. Once again, in the tenth week, which marked the transition after a challenging condition, i.e., removal of Tylan® from feed and reassignment to new finishing rooms with litter groups broken up, showed a major disruption in the clustering pattern with samples falling into six different groups. The sampling times corresponding to weeks twelve and sixteen, a time of stability for the piglets, once again showed coalescing of the microbiota; the twelfth week samples clustered into three groups, while the sixteenth week samples all clustered into a single group. Finally, for the last sampling period corresponding to the nineteenth week, also a transitional time after a challenge, i.e., movement to new finishing rooms with re-assortment of the groups, the samples once again showed a split into three different groups. The clustering pattern also showed that as the pigs aged, most samples clustered with

tonsillar samples from sows, despite no longer having contact with the sows. Based on the above analysis we identified three sampling times (third, eighth and sixteenth weeks), which were immediately before a challenging condition, where the microbiome tended to be more similar between pigs. Statistical support for this clustering pattern is shown in an unrooted dendrogram based on Bray-Curtis analysis (Figure 3.3.), where samples derived from the third, eighth and sixteenth weeks formed three distinct groups which were supported by bootstrap values higher than 70.

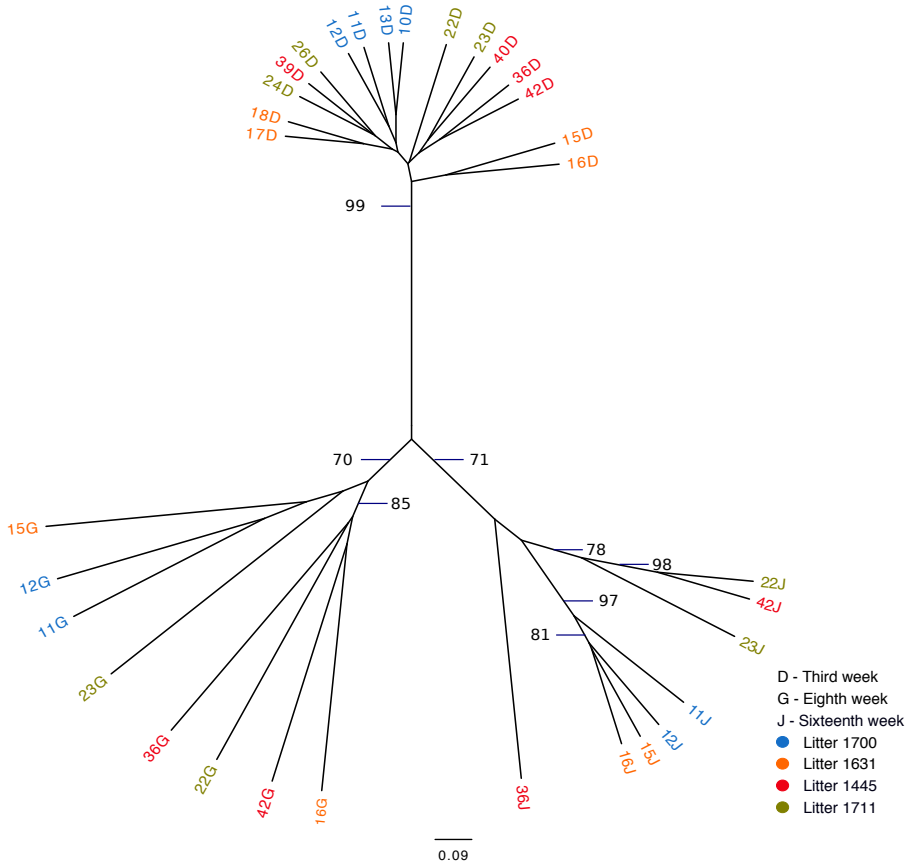

Figure 3.3. Unrooted Bray-Curtis dendrogram for three pre-transition times . The dendrogram shows the clustering of tonsil microbiome samples from pigs at three times immediately before challenging events: week 3, week 8, and week 16. Samples are color coded by week of sampling. Bootstrap values higher than 70 are shown.

We also analyzed the clustering shown in Figure 3.2. to determine whether there were effects of litter or of pen on the clustering. Samples from newborn and 1 week old pigs clustered by litter, but older animals did not. We saw no correlation of the clustering with groups in the same pens except as related to the litter effect seen in newborn and 1 week old animals

Tonsil microbiome membership throughout the life of the pigs. To visualize how the membership of the tonsillar microbiome changes through the life of the pigs, we plotted the proportion of the 20 most commonly identified bacterial families in piglets at each sampling time, as well as in sows (Figure 3.4.). Members of the phyla *Actinobacteria* (Family *Micrococcaceae)*, *Bacteroidetes* (Families *Bacteroidaceae*, *Porphyromonadaceae*, *Prevotellaceae*, *Flavobacteriaceae*), *Firmicutes* (Families *Bacillaceae 1*, *Staphylococcaceae*, *Streptococcaceae*, *Clostridiaceae 1*, *Clostridiales Incertae Sedis XI*, *Lachnospiraceae*, *Peptostreptococcaceae*, *Ruminococcaceae*, *Erysipelotrichaceae*, *Veillonellaceae*), *Fusobacteria* (Family *Fusobacteriaceae)*, and *Proteobacteria (*Families *Burkholderiaceae*, *Neisseriaceae*, *Pasteurellaceae* and *Moraxellaceae*) were identified as the most abundant bacterial phyla and families in pig tonsils. The distribution and proportions of these bacterial families fluctuated through the sampling period (Table 3.3.), with the largest shifts related with challenging conditions experienced by the pigs. Three families that consistently represented a major portion of the tonsil microbiome across all time points were the *Streptococcaceae, Pasteurellaceae,* and *Moraxellaceae.*

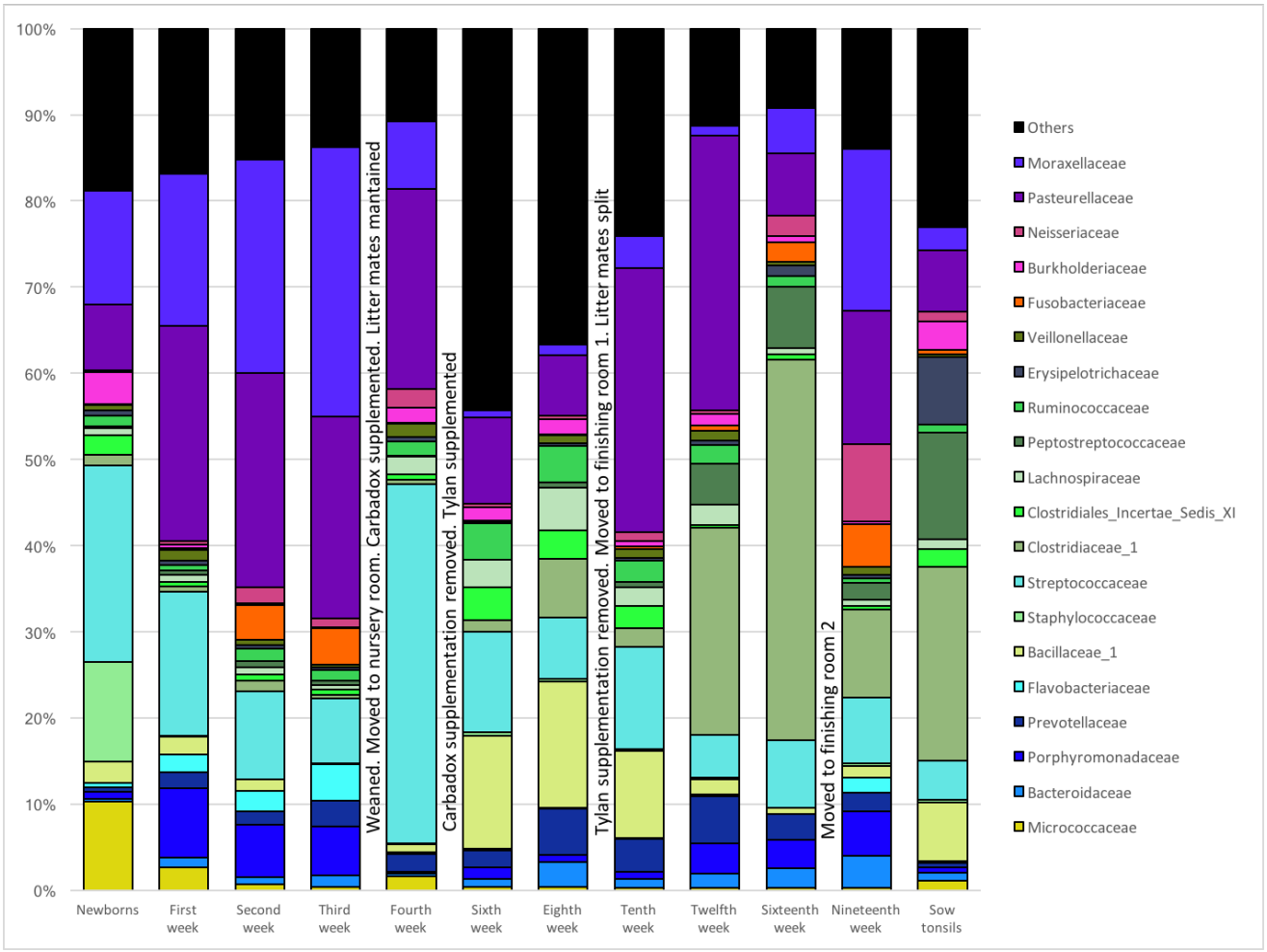

Figure 3.4. Twenty most abundant families identified in the tonsillar microbiome of pigs from newborn to market age and in the sows. The chart shows the twenty most abundant families identified in the tonsillar microbiome of the sampled

Figure 3.4 (cont´d). piglets from newborn through market age as well as the sows. Text boxes in the plot indicate times when a challenging event occurred. “Others” represent members of bacterial families different from the 20 most abundant families identified.

The microbiome of newborns was characterized by the abundant presence of the families *Streptococcaceae*, *Moraxellaceae*, *Staphylococcaceae* and *Micrococcaceae*, each representing 10 to 23% of the total; members of families *Pasteurellaceae*, *Burkholderiaceae* and *Bacillaceae* as well as members of the order *Clostridiales* were identified in smaller proportions. In the first week, *Pasteurellaceae* and *Porphyromonadaceae* increased dramatically, to 25% and 8.1%, respectively. *Moraxellaceae* also increased slightly, while there was a slight decrease in *Streptococacceae.* A more dramatic decrease was evident for *Staphylococcaceae,* which almost disappeared, and *Micrococcaceae*. Over the next two weeks, members of the *Streptococcaceae* continued to decrease, and *Micrococcaceae* virtually disappeared. In contrast, members of *Moraxellaceae* continued to increase. Members of *Pasteurellaceae* remained constant. *Fusobacteriaceae* appeared in week 2 and remained present in week 3. Multiple members of the order *Clostridiales* (*Clostridiaceae 1*, *Clostridiales Incertae Sedis XI*, *Lachnospiraceae*, *Peptostreptococcaceae* and *Ruminococcaceae*) were present in proportions lower than one percent, each, throughout the first 3 weeks of life in these piglets.

The transition between the third and fourth weeks, when the piglets were weaned and shifted to solid food containing Carbadox®, was marked by drastic shifts in the tonsil microbiome. *Moraxellaceae* decreased dramatically from 31.2 % in week 3 to 7.9 % in week 4, *Streptococcaceae* bloomed from 7.4 % to 41.6 %, while *Pasteurellaceae* and *Clostridiales* remained steady. Members of *Fusobacteriaceae* and *Porphyromonadaceae* almost disappeared.

Week 6, after another major transition when Carbadox® was removed from feed and Tylan® added, was again marked by drastic shifts in the tonsil microbiome. Overall sobs, as described above, increased from 111 to 368, indicating a massive increase in diversity. Members of the *Streptococcaceae* and *Pasteurellaceae* both decreased dramatically, from 41.6% to 11.6% and 23.2 % to 10%, respectively, and *Moraxellaceae* decreased and almost disappeared. However, members of *Bacillaceae 1* and some members of the order *Clostridiales* (*Clostridiales Incertae Sedis XI*, *Lachnospiraceae* and *Ruminococcaceae*) began to flourish and increased substantially, particularly *Bacillaceae* 1 which increased from 0.9 to 13.1%. Interestingly, almost 44% of the members of the tonsillar microbiome did not fit into these twenty most abundant bacterial families for this time point.

In the eighth week, the decreasing trend for *Streptococcaceae* and *Pasteurellaceae* continued and each family dropped to a relative abundance of 7%. *Moraxellaceae* remained in very low abundance. However, *Clostridiales,* particularly *Clostridiaceae 1*, increased, as did *Bacteroidales.* The proportion of identified bacterial families that were not included in the twenty most abundant was still close to forty percent.

The tenth week, which corresponded to another significant transition period for the pigs, i.e., removal of Tylan® from feed as well as movement to finishing rooms and reassortment of litter members, was again marked by a major shift in the microbiome. The three predominant families, *Pasteurellaceae, Streptococcaceae,* and *Moraxellaceae,* all increased, particularly the *Pasteurellaceae* that increased from 7% to 30.7%. In contrast, members of the *Clostridiales* (*Clostridiaceae 1*, *Clostridiales Incertae Sedis XI*,

Lachnospiraceae and *Ruminococcaceae*) and *Prevotellaceae* decreased, as did the proportion of the microbiome classified as "Others".

Over the next 6 weeks, represented by sampling times at 12 weeks and 16 weeks, the tonsil microbiomes in all of the pigs coalesced to a common core (Figure 3.2.). Overall, there was a massive increase in the *Clostridiales,* particularly *Clostridiaceae 1* and *Peptostreptococcaceae*, from 2.2% and 0.6% in week 10 to 44.1% and 7%, respectively in week 16. Over the same period, *Pasteurellaceae* decreased from 30.7% to 7.1%, and *Bacillaceae 1* decreased from 10.1% to 0.6%. *Streptococcaceae, Moraxellaceae,* and *Bacteroidales* remained relatively stable. The proportion of identified bacterial families that were not included into the twenty most abundant families decreased to ~10%. By week 16, *Fusobacteriaceae* and *Neisseriaceae* reappeared in low proportions.

Finally, the nineteenth week, which coincided with a transitional period in which penmates were reassorted into new rooms, was marked by another significant disruption of the microbiome. Overall, an increase in *Pasteurellaceae, Moraxellaceae, Neisseriaceae* and *Fusobacteriaceae* was paired with a dramatic decrease in *Clostridiales*, particularly *Clostriaceae 1* and *Peptostreptococacceae*.

It should be noted that, after the first 3 weeks and weaning, these shifts in the microbiome were not synchronous in all piglets. Figure 3.2. shows several clusters that contain samples from sequential weeks, e.g., weeks 6, 8 and 10; weeks 8, 10, and 12; and weeks 10, 12, and 16, indicating that common microbiomes, represented by the clusters, were reached at different times in different pigs. This is further illustrated in Figure 3.6. which shows the most abundant microbial families over time in 4 different

pigs. As examples, a microbiome with a preponderance of *Pasteurellaceae* is seen in week 10 in pig 23, week 12 in pig 11, both weeks 10 and 12 in pig 22, and not at all in pig 36. A microbiome with a preponderance of *Clostridiaceae*, mainly *Clostridiaceae 1* and *Peptostreptococcaceae*, is seen in weeks 12 and 16 in pig 36, week 16 in pigs 22 and 23, and weeks 16 and 19 in pig 11.

The tonsillar microbiome of sows was dominated by members of *Clostridiaceae 1* (~23%) and *Peptostreptococcaceae* (~12%). Other families present in proportions between 1 and 8% included *Erysipelotrichaceae*, *Pasteurellaceae*, *Bacillaceae 1*, *Streptococcaceae*, *Burkholderiaceae*, *Moraxellaceae*, *Neisseriaceae*. *Micrococcaceae* and other members of the *Clostridiales* (*Clostridiales Incertae Sedis XI*, *Lachnospiraceae* and *Ruminococcaceae*).

Distribution of specific OTUs throughout the life of the pigs. While presentation of the microbiome data at the taxonomic level of family gives the best overview of the data over time, we also examined the presence and abundance of specific OTUs over time (Figure 3.5.). At the family level, *Pasteurellaceae*, *Streptococcaceae,* and *Moraxellaceae* predominate throughout the life of the pigs. However, within the top 40 OTUs there were three OTUs of *Pasteurellaceae* seen, including OTU0001, which was present in high concentration during weeks 1-4 but never lost, OTU0016 which appeared in weeks 6, 10-12; and OTU0031, which was mainly seen in one week old piglets. Similarly, there were three OTUs of *Streptococcaceae,* including OTU002 which was seen throughout the lives of the pigs, OTU009 which was seen in newborns and weeks 1-4, and OTU0024, which was seen mainly in older piglets. Finally, there were three OTUs of *Moraxellaceae*, including

OTU003, which was a major component of the microbiome in newborns through week 4 and then disappeared to return in the week 19 samples; OTU0006, which was present in lower amounts than OTU0003 in weeks 1-4 but in much higher amounts in weeks 16 and 19; and OTU0046, which was a minor component of the microbiome.

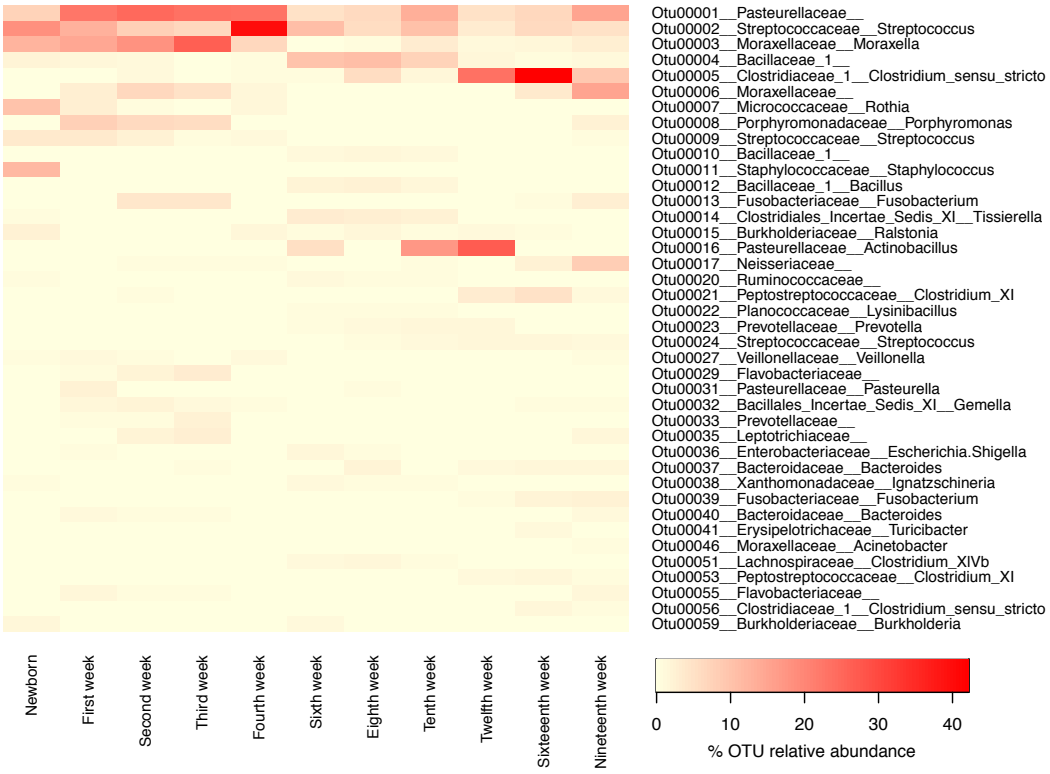

Figure 3.5. Forty most abundant Operational Taxonomic Units (OTUs) for pigs through different sampling times. Heat-map showing the relative abundance of the top 40 OTUs identified per sampling time through the life of these pigs. Note that any OTUs identified as "unclassified" at the family level were not included in this list.

Aerobic, anaerobic, and facultatively anaerobic organisms in the tonsils. An analysis of the distribution of the bacterial families identified in the tonsils based on

their classification by use of oxygen as aerobes, anaerobes or facultative anaerobes (Figure 3.7) showed that in piglets aged newborn to 4 weeks the microbial population was comprised of ~70% aerobes and facultative anaerobes. The abundance of facultative anaerobes decreased from birth through week 3, but increased after weaning, most likely due to the bloom in *Streptococcaceae*. The proportion of anaerobes increased after the weaning period, with a concomitant decrease in facultative anaerobes and aerobes, and reached ~65% of the total microbiome in week 16.

DISCUSSION

We have previously characterized the tonsil microbiome in healthy 18-20 week old grower-finisher pigs [12] and sought in this study to characterize how that tonsil microbial community develops during the life of pigs from newborn to market age. In particular, we wished to determine when specific members of the tonsil microbial community appeared, and whether there was a temporal succession in the development of the community.

There are strong parallels between our current data and that from the prior study [12]. In both studies, members of the tonsil microbiome were found to predominantly belong to 5 phyla: *Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria,* and *Actinobacteria*, with *Proteobacteria* and *Firmicutes* together representing ~85-90% of the tonsil microbiome. In both studies, *Pasteurellaceae, Moraxellaceae, Neisseriaceae, Streptococcaceae, Peptostreptococcaceae, Veillonallaceae,* and *Fusobacteriaceae* were identified as among the most abundant bacterial families found. In the current study, many families in the orders *Clostridiales* and *Bacteroidetes* were also found to be among the most abundant taxa seen. Improvements in both the sequencing technology and the databases that facilitate identification of bacteria via 16s rRNA gene sequencing likely account for these differences. In the earlier study, it was not possible to identify most *Clostridiales* below the order level, which is now possible. Further, in the earlier study it was recognized that *Bacteroidetes* were underrepresented in the final data, possibly due to amplification bias with the primers used in that study [12].

We collected samples from eleven different sampling periods from newborn to 19 weeks of age (Figure 3.1.) as well as the tonsillar microbiome of the sows. In our analysis of the taxa (at the family level and the OTU level) in these samples, we observed that the development of tonsillar communities in pigs followed a successional process. Some members of the community were acquired very early in life, from the sow vaginal tract and the teat skin or milk [22], while others were acquired later. Some members of the community, particularly *Streptococcus, Pasteurellaceae*, and *Moraxella*, were present throughout the life of the pigs, while others such as *Staphylococcus* and *Fusobacterium* seemed to be transient (Figure 3.4.). Further, specific OTUs of some of these major taxa also appeared to be either permanent or transient (Figure 3.5.). The relative proportions of the major members of the microbiome did change through time, however, as pigs aged, their microbiome seemed to become more similar to the microbiome of older pigs (Table 3.3.). This process was not always synchronous, but the overall progression was very similar in most pigs.

As we examined the changes in the microbiome over time, it became clear that at certain time points, e.g., at 3, 8, and 16 weeks, the microbiomes of all of the animals became very similar, or coalesced (Figure 3.2. and 3.3. and Table 3.2.). When we analyzed this in comparison to management of the pigs (Figure 3.1.), we concluded that stretches of time with constant conditions, such as newborn through week 3, led to this coalescing of the microbiome. This coalescing occurred regardless of litter source for the pigs or room in which they were housed. In contrast, times where there were changes in management conditions, such as addition or removal of in feed growth promoter antibiotics or movement of pigs to new housing, and especially weaning, led

to perturbations in the microbiome (e.g., weeks 4, 6 ,10 and 19. The taxonomic data was supported by an analysis of the alpha diversity (sobs: number of observed OTUs, Table 3.2.). Whether these perturbations occurred in response to specific stresses, such as presence of antibiotics, or were adaptations of the microbiome to new conditions, such as new feed, remains unclear.

There are no studies available that describe the development of the tonsillar microbiome of pigs or other mammals, except this study and one recently submitted by our lab that followed the development of the tonsillar microbiome of piglets from newborn to weaning, focusing on the source of members of the microbiome and the litter effect as well as the overall development and the effect of weaning [22]. Most of the available data following the development of microbial communities in mammals has been focused on the gastrointestinal tract. Pajarillo et al [29] assessed the fecal bacterial diversity of healthy piglets during the weaning transition, and suggested that this period was related to a trend of increasing bacterial diversity, which may be related with the changes in diet. However, they did not discard a possible additional influence of stress or disruption associated with the weaning period. Another study describing the bacterial diversity of pig feces over time followed the development of fecal microbiome from pigs, between 10 to 22 weeks old, and identified that calculated diversity indices suggested similar diversity profiles for all the samples [30]. Although these prior studies examined the fecal microbiome, they support our results of increased bacterial diversity when the piglets were weaned, which decreased after 10-12 weeks, following challenges or disruptions, such as addition or removal of in feed antibiotics.

It should be noted that the shifts in the membership and diversity of the tonsil microbiota that we observed were often associated with challenges that occurred in several combinations and rarely were associated with a singular change in the management of the pigs. Changes in diet, the administration of antibiotics, and the environment frequently occurred simultaneously, making it difficult to determine which challenges were associated with the observed microbiota shifts. However, absence of challenges or disruptions led to stabilization of the microbiome, with most pigs developing similar microbiomes over times with constant conditions.

There is extensive research data showing that that the balance of microbial communities is altered by the use of antibiotic treatments [20, 31]. Rettedal et al [32] studied the effect of the growth promoter chlortetracycline on the ileal microbiota of pigs and found that use of this antibiotic was associated with a significant shift in the gut microbiota, including a replacement of the dominant species of *Lactobacillus* and decrease in relative abundance of *Turicibacter* . However, Poole et al [33] did not find changes in diversity in feces associated with a similar dose of chlortetracycline supplementation . The in-feed supplementation of pigs with a mixture of antibiotics known as ASP250, containing chlortetracycline, sulfamethazine and penicillin, was correlated with a shift in the bacterial phylotypes present in the intestine, where microbial community membership changed over time associated with administration of the product. The changes were related mainly with a decrease in *Bacteroidetes* abundance and an increase in *Proteobacteria*, among other changes [34]. Carbadox® supplementation in-feed was associated with significant changes in community structure and bacterial membership in the intestinal microbiota of pigs. An immediate

effect was noticed in the bacterial community after the administration of this drug, although the microbiome structure recovered later despite the continued use of the medication. The authors reported a relative increase in *Prevotella* associated with Carbadox® administration, while Carbadox® withdrawal was associated with an increase in the *E. coli* population [35]. The use of the growth promoter Tylosin (also known as Tylan®) was associated with a pronounced shift in the intestinal microbiome distribution and quantity, altering the abundance of specific genera such as *Lactobacillus* among others. These changes occurred at specific times in the growing pig as the pigs aged [36]. These prior studies have tried to identify the effects of medicated food on the gut/feces microbiome, but there are no studies that characterize the effect of ingested medications on the tonsillar microbiome. However, it can be concluded that regardless of the medication, the administration of antibiotics or growth promoters in food exerts an effect on the bacterial communities. In this study, we observed large shifts in the tonsil microbiome related to specific periods where medicated food was added, changed or removed. However, because in feed medication was not an isolated factor but supplementary to other changes at the same time, we cannot make a definitive conclusion about the specific effect of the administration of this medication. We do consider the microbiome shifts seen to be relevant and the potential subject of further research.

We identified the first major shift associated with supplementation of Carbadox® coinciding with a huge bloom in members of *Streptococcaceae* and a decrease in *Moraxellaceae, Fusobacteriaceae* and *Porphyromonadaceae*, reported previously by our group [22]. Another shift was associated with the removal of Carbadox® and

supplementation with Tylan®, with a major decrease in members of *Streptococcaceae*, *Moraxellaceae* and *Pasteurellaceae* with a concurrent increase in members of *Clostridiales* and *Bacillaceae 1*. The removal of Tylan® from the diet was associated with a slight increase in members of *Streptococcaceae* and *Moraxellaceae*, parallel to a higher increase in members of *Pasteurellaceae*. We believe it is relevant to highlight that the presence of Tylan® in the diet is associated with an increase in the bacterial diversity (Table 3.2. and Figure 3.4.). We emphasize that our goal was not directed towards the identification of specific effects of antibiotics or growth promoters in the development of the tonsil microbiome, but instead towards characterization of the development of tonsillar microbiome of pigs from a healthy farm. Our results open an avenue for future research on the specific effect of these medications on the tonsillar microbiome and how they can potentially influence the acquisition of pathogenic flora.

Another big change experienced by the pigs was dietary, particularly at weaning. In human infants, the introduction to a new diet associated with cessation of breast feeding has been shown to be associated with profound changes in the composition of the intestinal microbiome [37]. It has been suggested that the diet to which an individual has been exposed rapidly alters the structure of the intestinal microbial communities [38]. Similarly, in pigs it has been shown that the diet can have an effect in the intestinal microbiome [39], and in particular that the diet supplemented after weaning in piglets can alter the fecal microbiota considerably. A diet supplemented with fermentable carbohydrates was related with greater bacterial diversity when compared to control diets [39]. Diet changes during weaning transition can exert an

effect on the composition of the intestinal microbiota [40, 41] where bacterial community structure can change as the diet changes [42].

Finally, the environmental changes experienced by the pigs could play a role in the development of the tonsillar microbiome. These can include both changes in the physical environment and exposure to new penmates after reassortment of pigs into new housing. The immediate environment in which pigs grow has been suggested to have a profound influence on the initial acquisition and development of fecal and colonic microbiota [43]. A recent study following the development of gut microbiota and the effect of early changes in the environment demonstrated that microbial diversity was disturbed by changes in environmental hygiene, and that the effect of the generated changes remained for a long time in the affected animals [8]. In our study, we saw increased fecal anaerobes, such as *Clostridiales,* in the tonsils (Figure 3.4. and Figure 3.7.) after weaning and especially after Tylan® was removed from feed. Pigs are coprophagic, and it is likely that these anaerobes were acquired from ingestion of feces from the pen floors. In the older pigs, crypt abscesses in the deeper areas of elongating crypts might provide a niche for colonization by the acquired anaerobes, or conversely these anaerobes may cause the formation of the crypt abscesses. We have previously observed that pigs housed in a very clean high biosecurity environment had almost no *Clostridiales* in the tonsils (unpublished data).

The sixteenth week constituted the highest threshold for members of *Clostridiales* with *Clostridiaceae 1* and *Peptostreptococcaceae* comprising ~51% of the identified members of the microbiome for this period. Our results are supported by Bokulich et al [31], who studied the development of fecal microbiota in children during

early life and associated the administration of antibiotics in children during first months of life with deficit in members of *Clostridiales*. Further, the authors associated a gradual increase in members of this order with the introduction to solid food. Our findings become especially relevant when compared with recent findings reported by Kim et al [7], which found that the presence of members of *Clostridiales* in the enteric microbiota of mice is critical to prevent the growth of enteric pathogens in the intestine. We do not know how this finding can be translated to the tonsillar microbiome of pigs, but it is interesting to see that one of the most vulnerable periods for pigs to acquire diseases (weaning through eighth week) was marked by a low abundance of members of the *Clostridiales*.

We need to emphasize that the goal of this research was not to examine the individual effect of each one of the possible factors associated with the challenges experienced by the pigs during their lives but on the contrary, the purpose of this work was to characterize the development of porcine tonsillar microbiome of healthy pigs from newborn through market age under normal management conditions and thus to create a base knowledge in a topic so far little studied.

In this study, we found that there were some bacterial families that dominated the tonsillar microbiome throughout the life of pigs; however, their relative abundance often changed significantly after the challenging events. Similarly, other bacterial families appeared and/or disappeared at specific ages. We identified that members of *Pasteurellaceae*, *Streptococcaceae* and *Moraxellaceae* were the most abundant families through the life of pigs despite their fluctuation at certain ages. Nevertheless, *Pasteurellaceae* was the most abundant family throughout the study period. The greater

presence of members of *Clostridiales* particularly *Clostridiaceae 1* and *Peptostreptococcaceae* in older pigs needs to be highlighted, as well as the sudden increase in members of *Fusobacteriaceae* at certain ages. A longitudinal study of bacterial diversity in feces of commercial pigs found that some phyla dominated the microbiome regardless of the age of the animals, supporting our findings in the tonsils. Further, it was observed that a small group of organisms were the most prevalent microbes as pigs aged, and their microbiome converged with the time when they were maintained under similar conditions [30]. Although this study was focused on the fecal microbiome, it supports our results in the development of tonsillar microbiome, where we identified some bacterial families that dominated and were present throughout the study period, as well as other bacterial families that were transient and appeared at different times, and further saw a convergence of the tonsil microbiome in all the pigs when they were maintained under constant conditions.

Jensen et al [11] characterized the microbiome of tonsillar crypts of human patients either with chronic tonsillitis or tonsils from healthy patients which were removed because of hyperplasia. The authors could identify a core microbiome population at the species level in the crypts of humans independent of their health status and age, which involved the genus *Streptococcus*, *Prevotella*, *Fusobacterium*, *Porphyromonas*, *Neisseria*, *Parvimonas*, *Haemophilus*, *Actinomyces*, *Rothia*, *Granulicatella* and *Gemella*. The above identified genera are members of the families *Streptococcaceae*, *Prevotellaceae*, *Fusobacteriaceae*, *Porphyromonadaceae*, *Neisseriaceae*, *Clostridiales Incertae Sedis XI*, *Pasteurellaceae*, *Actinomycetaceae*, *Micrococcaceae*, *Carnobacteriaceae* and *Bacillales Incertae Sedis XI*, respectively.

Similarly, other studies identifying the human microbiome, recognize members of families *Streptococcaceae*, *Prevotellaceae* and *Fusobacteriaceae* as abundant in the tonsillar microbiome of healthy humans [13, 16, 44]. Although we did not characterize specifically the microbiome of tonsillar crypts and we were not able to characterize the members of the community further than family or genus level for some taxa, our results also show that members of the above mentioned families except *Actinomycetaceae* and *Carnobacteriaceae,* comprised some of the most abundant families identified in pig tonsils. It was found that members of the genus *Staphylococcus* were present only in low proportions in human tonsils [11]. Similarly, we identified that members of the family *Staphylococcaceae* were abundant only in the newborns and decreased noticeably and almost disappeared on the following weeks.

A Bray-Curtis analysis of the development of pigs microbiome from birth to market age (Figure 3.2.) showed us that as the pigs were getting older, the acquired microbial population tended to be more similar to the microbiome present in adult pigs, i.e., the tonsillar microbiome of sows (Figure 3.4). We identified that between the sixth to tenth week, some samples clustered with a sample from the tonsillar microbiome of sows. However a higher percentage of samples from older pigs, especially between twelfth to nineteenth weeks, were clustered together with samples from tonsillar microbiome of sows. These findings demonstrate both that there is a succession in the development of tonsillar microbiome in pigs and that the final status of the microbiome in grower/finisher pigs develops to resemble that of adult animals. Similar findings were reported by other authors studying the development of the human intestinal microbiota [19, 31], which found that as infants aged, their gut microbiome began to

look like the adult microbiome, although it did not reach a mature stage found in adults. Our results shows that although the microbiome of older pigs was more similar to the microbiome of the sows, there are still observable differences in the abundance of certain families, as the case of members of families *Peptostreptococacceae*, *Erysipelotrichaceae* and *Burkholderiales* which were more prominent in sow microbiome. Many other studies have also shown that there is a succession/sequentiality in the development of microbial communities in mammalian tissue [17, 18, 30, 45, 46]

CONCLUSIONS

This study provides baseline information on the development of tonsillar microbiome of piglets from newborn to market age, as well as the tonsillar microbiome of sows. We demonstrate that there was a succession in the development of the tonsillar microbiome of piglets as they age, which was not synchronous on all pigs but was highly similar. The tonsil microbiome tended to stabilize and become very similar in all animals over times where management conditions are constant. However, the challenges associated with management procedures typical in a swine farm generated prominent changes in the microbiome composition and the abundance of diverse bacterial families. Nonetheless, over time the microbiome of these young pigs tended to be more similar to the microbiome of older. We do not know if the observed patterns would be similar for all pigs from this farm, or if the same pattern would be observed independent of the breed or the specific farm. This study lays the baseline for future

research to examine the effect of specific conditions, such as use of antibiotics, on the development of the tonsil microbiome and of acquisition of specific pathogens on the tonsil microbiome and conversely of the effect of the composition and structure of the tonsil microbiome on acquisition of pathogens. Manipulation of the tonsil microbiome to provide enhanced resistance to acquisition and carriage of pathogens is a potential outcome of these studies.

APPENDIX

Table 3.3. Top 20 most abundant families per sampling time

Row Labels	Newborns	First week	Second week	Third week	Fourth week	Sixth week	Eighth week	Tenth week	Twelfth week	Sixteenth week	Nineteenth week	Sow tonsils
Corynebacteriaceae	2.02	0.14	0.07	0.07	0.22	0.68	0.68	0.28	0.10	0.25	0.30	1.50
Micrococcaceae	10.25	2.63	0.74	0.42	1.68	0.43	0.38	0.29	0.30	0.23	0.26	1.11
Bacteroidaceae	0.30	1.14	0.74	1.33	0.29	0.93	2.94	1.07	1.60	2.33	3.78	0.93
Porphyromonadaceae	0.85	8.11	6.11	5.69	0.21	1.34	0.78	0.81	3.54	3.34	5.11	0.59
Prevotellaceae	0.51	1.79	1.58	2.97	2.05	1.90	5.40	3.75	5.52	2.92	2.21	0.55
Rikenellaceae	0.24	0.07	0.02	0.00	0.04	1.16	0.54	0.70	0.04	0.02	0.11	0.00
Flavobacteriaceae	0.59	2.06	2.38	4.22	0.18	0.25	0.11	0.17	0.10	0.08	1.67	0.18
Chitinophagaceae	0.17	0.01	0.00	0.01	0.04	0.15	0.10	0.14	0.56	0.28	0.04	0.11
Bacillaceae_1	2.40	2.10	1.29	0.13	0.94	13.13	14.60	10.07	1.83	0.65	1.37	6.83
Bacillales_Incertae_Sedis_XI	0.19	1.32	1.93	1.22	0.51	0.06	0.14	0.09	0.04	0.44	0.82	0.76
Planococcaceae	0.15	0.16	0.10	0.03	0.10	0.82	0.88	0.65	0.08	0.24	0.11	1.25
Staphylococcaceae	11.60	0.07	0.06	0.03	0.08	0.34	0.30	0.25	0.19	0.08	0.36	0.35
Aerococcaceae	0.36	0.57	0.18	0.07	0.09	0.00	0.00	0.04	0.17	0.33	1.23	0.15
Lactobacillaceae	0.16	0.60	0.31	0.03	2.25	0.55	0.21	0.19	0.23	0.22	0.06	0.08
Streptococcaceae	22.77	16.71	10.23	7.45	41.64	11.63	7.09	11.86	4.90	7.83	7.58	4.56
Clostridiaceae_1	1.30	0.67	1.21	0.49	0.58	1.35	6.90	2.21	24.10	44.14	10.19	22.48
Clostridiales_Incertae_Sedis_XI	2.25	0.49	0.73	0.59	0.59	3.82	3.29	2.48	0.32	0.56	0.43	2.03
Lachnospiraceae	0.78	0.87	0.85	0.49	2.06	3.21	4.92	2.18	2.30	0.77	0.72	1.17
Peptostreptococcaceae	0.24	0.45	0.67	0.50	0.11	0.08	0.65	0.63	4.75	7.09	1.97	12.31
Ruminococcaceae	1.21	0.67	1.41	1.24	1.64	4.18	4.19	2.52	2.17	1.21	0.53	1.00
Erysipelotrichaceae	0.69	0.47	0.41	0.32	0.54	0.06	0.29	0.25	0.53	1.33	0.39	7.77
Veillonellaceae	0.59	1.28	0.61	0.28	1.57	0.27	0.93	1.05	1.20	0.39	0.95	0.39
Fusobacteriaceae	0.09	0.23	4.11	4.29	0.12	0.01	0.13	0.34	0.54	2.28	4.96	0.49
Leptotrichiaceae	0.00	0.00	2.58	3.55	0.00	0.06	0.01	0.00	0.01	0.16	1.88	0.49
Caulobacteraceae	2.20	0.05	0.02	0.07	0.26	0.06	0.07	0.14	0.04	0.00	0.00	0.48
Sphingomonadaceae	0.31	0.10	0.01	0.03	0.31	0.52	0.51	0.33	0.19	0.03	0.20	0.98
Burkholderiaceae	3.73	0.38	0.13	0.08	1.68	1.54	1.73	0.62	1.39	0.69	0.35	3.32
Comamonadaceae	0.75	0.17	0.11	0.02	0.27	0.97	1.11	0.73	0.40	0.18	0.04	0.98
Neisseriaceae	0.17	0.43	1.87	1.07	2.17	0.36	0.48	1.01	0.40	2.39	8.90	1.08
Succinivibrionaceae	0.04	0.01	0.02	0.00	0.05	0.05	1.45	0.03	0.06	0.02	0.00	0.14
Enterobacteriaceae	0.93	0.49	0.25	0.04	0.49	2.32	1.10	0.86	0.37	0.43	0.27	0.78
Pasteurellaceae	7.68	24.90	24.93	23.40	23.25	10.00	7.02	30.70	31.87	7.16	15.47	7.17
Moraxellaceae	13.19	17.67	24.72	31.22	7.85	0.87	1.15	3.68	1.16	5.28	18.87	2.61
Pseudomonadaceae	0.12	0.06	0.01	0.01	0.09	0.33	0.71	0.45	0.91	0.03	0.00	0.38
Xanthomonadaceae	1.15	0.16	0.05	0.01	0.23	1.47	0.95	0.67	0.34	0.11	0.04	0.36
Spirochaetaceae	0.23	0.22	0.47	0.11	0.08	0.56	1.39	0.57	0.33	0.35	1.50	0.81
Others	9.80	12.76	9.07	8.51	5.71	34.54	26.88	18.22	7.43	6.15	7.33	13.84

The table shows the twenty most abundant families identified in the tonsillar microbiome of the sampled piglets from newborn through market age as well as the sows. The color code shows in red, the families that represents the top twenty most abundant families for a specific period or sample. The blue color represents families that were not part of the top 20 families for that period. In black color are other members of microbiome not shown in the table.

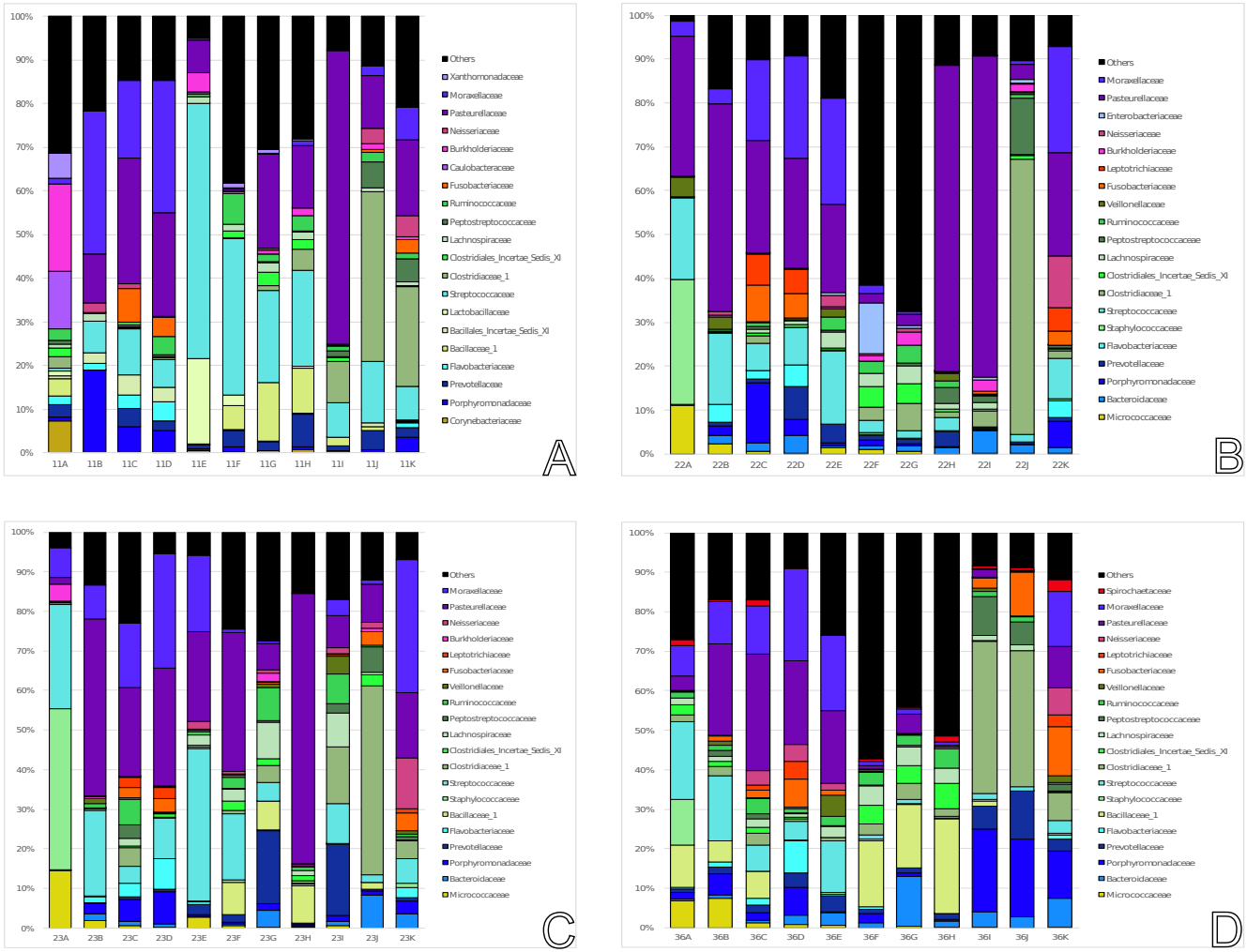

Figure 3.6. Top 20 most abundant families per sampling time for 4 selected pigs. Top 20 families through the time for pig 11 (A), pig 22 (B), pig 23 (C), pig 36 (D)

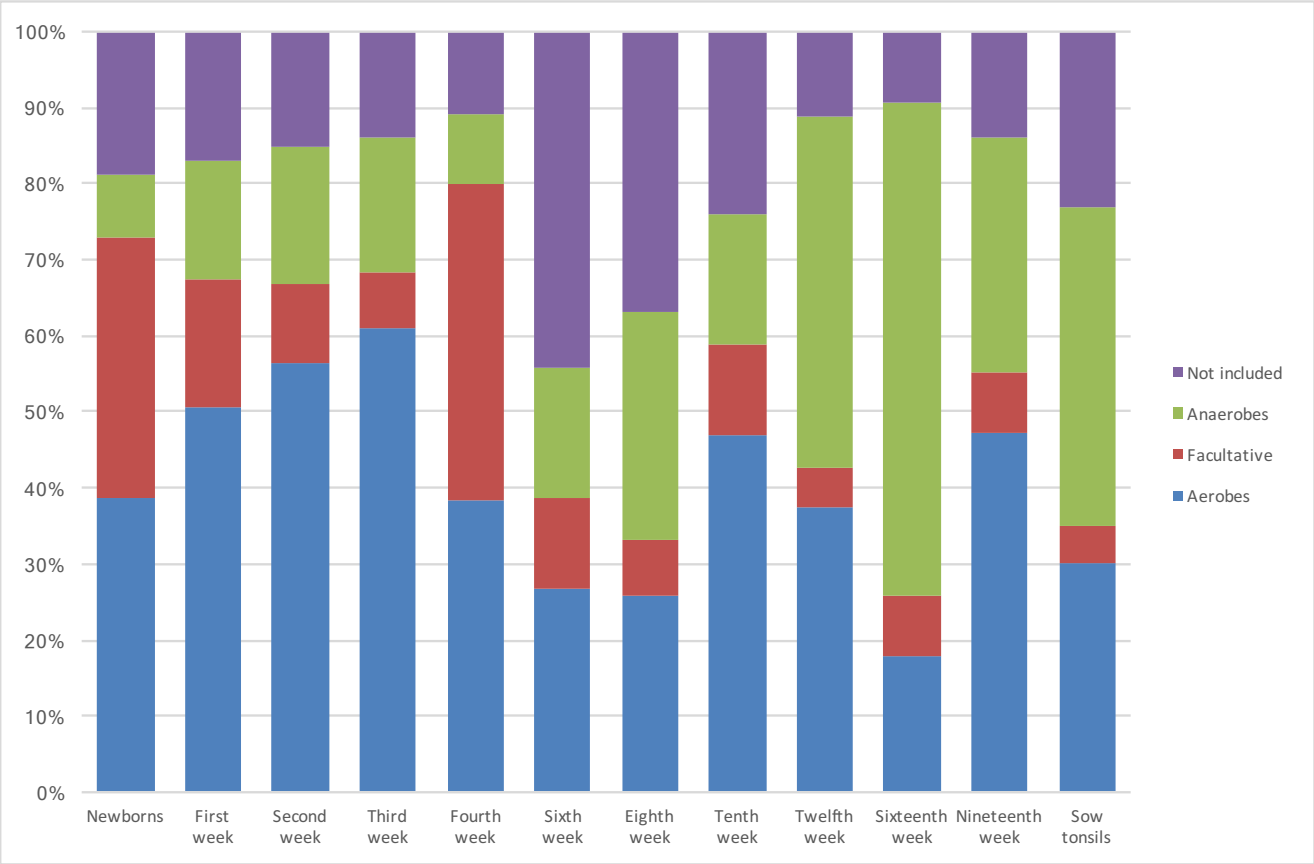

Figure 3.7. Proportions of aerobes, anaerobes and facultative bacteria. The plot chart shows the twenty most abundant families identified in the tonsillar microbiome of the sampled pigs from newborn through market age as well as sows and their proportion once classified as aerobes, anaerobes or facultative organisms based on Bergey´s Manual of Systematic Bacteriology [47]

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CHAPTER 4. SPATIOTEMPORAL DEVELOPMENT OF THE TONSIL MICROBIOME IN PIGS

INTRODUCTION

Tonsils are lympho-epithelial tissues strategically located at the junction of oropharynx and nasopharynx where they fulfill an important role in immune surveillance and protection of the host from pathogens gaining access via oral or respiratory routes [1]. Numerous host-specific and zoonotic pathogens such as bacteria and viruses can colonize tonsils and use them as a reservoir, generating a source that has high potential for transmission to other animals and to humans [2]. There is a paucity of data pertaining to the tonsillar microbiome in mammals. Further, there are no published studies about the development of the spatiotemporal structure of microbial communities of pig tonsils or tonsils of any other mammal.

Studies on the development of the spatiotemporal structure of microbial communities of other regions of the oral cavity, such as dental plaque in humans, have shown that a key characteristic of oral microbiome communities is the presence of a high diversity and temporal succession in their population [3]. In dental plaque, members of the genus *Streptococcus* function as early colonizers opening the way for attachment of other bacteria, creating the conditions suitable for the arrival of late colonizers which are frequently pathogenic bacteria [4]. Bacteria in the oral cavity, as well as in other tissues, have specific adherence molecules on their surface which are necessary for initiating colonization [4]. In the development of microbial communities of oral plaque, the community is structured spatially by the temporal succession of colonizing bacteria, where members of the genus *Streptococcus* are early colonizers and have receptors for co-aggregation of other early colonizers, such as members of

the genera *Actinomyces* and *Veillonella*. After the initial development of the microbiome of the human dental plaque, interbacterial cell-cell recognition plays a significant role in the spatiotemporal development of these communities [5].

After attachment of early colonizers to the dental surface, members of the genus *Fusobacterium* arrive and act as a bridge between early and late colonizers, which are frequently known pathogenic bacteria such as *Porphyromonas* and *Treponema*, among others [6]. An important characteristic of these associations is that there is no coadhesion or coaggregation between early and late colonizers; only *Fusobacterium* can coaggregate first with early and then with late colonizers [6].

Coaggregation is the cell-cell recognition between different types of bacteria [7]. It has been suggested that relationships between coaggregation partners lead to a temporal relationship with respect to colonization of surfaces. Studies done by Palmer et al. [5], using fluorescent labeled antibodies and confocal laser scanning microscopy (CLSM), for the first time showed the significance of coaggregation in the development of microbial communities and emphasized its importance in the spatiotemporal development of microbial communities.

The spatial distribution/localization of diverse bacteria has been studied in different tissues [8-13], using methods such as Scanning Electron Microscopy (SEM) [13], immunohistochemistry [9], and FISH (Fluorescence In Situ Hybridization) and detection of bacteria using epifluorescence and CLSM [8, 11, 13-16]. These techniques have helped understand the interplay of microbes in different tissues. However, limited knowledge is available about the spatial distribution, assembly and structure of tonsillar microbial communities in any mammal, including pigs.

Tonsils are colonized by bacteria from the genus *Streptococcus*, specifically *Streptococcus suis*, early in the lives of pigs, as early as during the birth process [17-19]. Studies using segregated early weaned pigs have suggested a temporality in colonization by other bacteria, such as *Haemophilus parasuis,* which were detected in the first day of life, followed by other bacterial species, such as *Pasteurella multocida* and *Bordetella bronchiseptica*, which arrive after the second week of life or later [17, 20]. Although there has been no comprehensive study of the patterns of tonsillar colonization by different bacteria or spatiotemporal structure of the bacterial communities in tonsils, the aforementioned studies suggest that there is a pattern of temporality in the building of tonsillar communities of pigs, at least in early life.

The goal of this study was to characterize how the three most abundant members of the pig tonsillar microbiome, *Pasteurellaceae*, *Streptococcaceaae* and *Moraxellaceae* [21, 22], are spatially localized at different times in the life of pigs. The use of FISH probes targeting bacterial 16S rRNA genes and CLSM allowed us to identify the presence of these different bacterial taxa in tonsils. Scanning Electron Microscopy (SEM) gave us a detailed view of the tonsils, their general appearance over time, and an overview of the organization of the microbial communities and how they developed over time.

MATERIALS AND METHODS

Animals. Approval for all experimental procedures was obtained from The Michigan State University Institutional Animal Care and Use Committee (IACUC).

Piglets derived from three crossbred sows (Yorkshire x Hampshire) from a high health status herd were used in this study. A detailed description of this herd has been published previously elsewhere [21]. One piglet from each of the three sows was selected randomly from the litter at each sampling time: 1, 3, 4, 8, 10 and 17 weeks. Pigs were initially anesthetized with a combination of Tiletamine-Zolazepam (Telazol®, Zoetis, USA) and Ketamine (Kethatesia, Henry Schein Animal Health, Dublin, OH), administered intramuscularly. Once anesthetized, they were euthanized by overdose of a pentobarbital solution (Fatal-Plus, Vortech Pharmaceuticals - Dearborn, MI), delivered intravenously into the vena cava, following standard procedures approved by IACUC.

Tissue collection. The entire right soft palate tonsil from each piglet was collected and the excess connective tissue removed using sterile scalpels and forceps. Each right tonsil was placed in a sterile glass Petri dish and divided into quarters. A random quarter of the tissue was carefully placed in a labeled disposable tissue cassette previously covered with a thin film of HistoPrep embedding medium (Fisher, Fair Lawn, NJ). Samples were oriented carefully to allow access to tonsillar crypts when trimming. Following this, the cassette was fully filled with the embedding media and immediately frozen at -80°C. After solidification, the sample blocks were removed from the cassettes, wrapped in aluminum foil, labeled and stored at -80°C until processing. Blocks were sectioned on a Leica Cryostat (Leica Microsystems, Vienna, Austria). Tissue sections (5 μm) were collected on positively charged microscope slides, Leica BOND™ Plus Slide (Leica Biosystems Richmond, Illinois, USA), and stored at -20°C until processing for FISH. Another random tonsil quarter was placed into a 15

ml sterile test tube containing 10 ml of 4% paraformaldehyde (PFA) for 4 - 6 h and then removed and stored in 50% ethanol until processed.

Bacterial strains and smears on slides. To validate the FISH probes, we used the following bacterial isolates: *Streptococcus suis*, *Staphylococcus aureus*, *Pasteurella multocida*, *Escherichia coli* (all from the laboratory collection of MHM) and *Moraxella bovis* (kindly provided by Dr. Rinosh Mani). Overnight cultures of BHI agar (BBL™ Brain Heart Infusion, Becton Dickinson and Company, Sparks, MD) streaked with each individual isolate were used to inoculate 5 ml of BHI broth to an initial optical density at 520nm (OD_{520}) of 0.15. Inoculated broths were incubated at 35°C shaking at 160 rpm until an OD_{520} of approximately 0.5 was reached. Bacterial suspensions were mixed with equal amounts of 4% paraformaldehyde (4% PFA) and fixed for 1.5 hours, followed by three washes in PBS, pH 7.4. Pellets were suspended in equal amounts of PBS and absolute ethanol and stored at -20°C in aliquots until used for validation of the probes in slide smears and muscle tissue.

Bacterial strains and muscle slides. To validate the FISH probes against bacterial cells present in mammalian tissue, 50 ml of overnight cultures of *Streptococcus suis*, *Pasteurella multocida*, *Escherichia coli* and *Moraxella bovis* were pelleted by centrifugation and suspended in 5 ml of sterile broth. Equal proportions of all the bacterial isolates were mixed in a sterile 10 ml tube and inoculated onto samples of muscle of approximately 3 x 3 x 10mm, which were collected aseptically and randomly from a euthanized pig used in the study. Inoculated muscle was processed in the same way as tonsil tissue.

Oligonucleotide probes. FISH experiments were carried out using the probe EUB338 [23] which targets a region of the bacterial 16S rRNA genes, present in most bacteria, and the nonsense probe NON338 [24], which is the antisense probe of EUB338 and was used to exclude non-specific probe binding. Specific nucleotide probes for the family *Pasteurellaceae* PAS111 [16], the genus *Streptococcus* STR405 [25] and the genus *Moraxella* MOR575 (designed in this study), were selected to target the three most abundant taxa identified in the tonsillar microbiome of pigs [21, 22] (Table 4.1.). The sequences for these FISH probes were obtained after a search of available journal publications or in probeBase [26] or using the probe design function of ARB [27], and compared with representative sequences for the respective OTUs, using the ribosomal database project (RDP) sequence analysis tools [28]. Probes were custom synthesized by IDT® (Integrated DNA Technologies, Coralville, IA).

Table 4.1. Oligonucleotide probes

Probe	Sequence (5′-3′)	Target genus or family	Fluorochrome	Labeled
EUB338	GCT GCC TCC CGT AGG AGT	Bacterial domain	Cy3	5′
EUB338	GCT GCC TCC CGT AGG AGT	Bacterial domain	Alexa 488	5′
NON338	ACT CCT ACG GGA GGC AGC	Antisense EUB338	Cy5	5′
STR405	TAG CCG TCC CTT TCT GGT	*Streptococcus*	Cy3	5′
PAS111	TCC CAA GCA TTA CTC ACC	*Pasteurellaceae*	Cy5	5′
MOR575	AAT GAC CAC CTA CGC TCG	*Moraxella*	Alexa 488	5′

Fluorescent In Situ Hybridization (FISH). For this procedure the probes were first validated against PFA-fixed pure cultures of the bacterial strains and mixtures of the strains spread on positively charged slides, Leica BOND™ Plus Slide (Leica Biosystems Richmond, Illinois, USA), and against samples of muscle tissue inoculated with a mix of bacterial cells as explained above. These slides were also

used as positive controls for probe hybridization and were included in all hybridization experiments. From every tonsil sample, four sequential sections were hybridized, each with a different set of probes. Section one was hybridized with the EUB338 and NON338 probes; section two was hybridized with the EUB338, MOR575 and PAS111 probes; section three was hybridized with the EUB338, STR405 and PAS111 probes; and finally a fourth section was hybridized with the MOR575, STR405 and PAS111 probes. Tissues were initially immersed in a PBS-Tween 20, pH 7.4 buffer, then incubated with a lysis buffer (100 μg/ml lysozyme in 0.1 mM Tris-HCl, pH 8.0) at 37°C for 10 min, serially washed with PBS-Tween buffer and then submitted to the hybridization protocol. A pre-warmed (46°C) solution containing 4 μl of each selected oligonucleotide probe (50 ng/μl) was mixed with hybridization buffer containing 0.9 M NaCl, 20 mM Tris-HCl, pH 8, 20% formamide (Fisher, Fair Lawn, NJ) and 0.01% SDS, to make up a volume of 100 μl per hybridized slide. The solution was applied on the tissue sections and incubated in a dark-humid histochemistry staining tray (IBI Scientific, Kapp Court Peosta, IA) for 3h at 46°C. Immediately following hybridization, slides were washed twice for 25 min at 48°C each, with pre-warmed (48°C) washing buffer containing 0.9 M NaCl, 20 mM Tris-HCl, pH 8, and 0.01% SDS. Finally, the slides were rinsed with ice-cold ultrapure water, gently dried with a paper towel and mounted with ProLong® Diamond antifade mountant with DAPI (Molecular Probes, USA). The mounting media was cured for at least 24 h at room temperature before analysis of the slides.

Confocal Laser Scan Microscopy (CLSM). A Nikon C2 Laser Scanning Confocal Microscope (Nikon Instruments Inc., Melville, NY) equipped with 405nm, 488nm,

561nm and 633 nm lasers was used to acquire the images. Image processing was performed with NIS Element viewer software (Nikon, version 4.1.11).

Scanning Electron Microscopy (SEM). Tissue samples stored in 50% ethanol were dehydrated in a series of graded ethanol solutions (50%, 75%, 95%) for 20 min at each gradation followed by three 10 min changes in 100% ethanol, dried by critical point drying with a Leica Microsystems model EM CPD 300 (Leica Microsystems, Vienna, Austria), mounted on aluminum stubs using carbon suspension cement (SPI Supplies, West Chester, PA) and adhesive tabs (M.E. Taylor Engineering, Brookville, MD), coated with iridium for 30 sec in a Quorum Technologies/Electron Microscopy sciences Q150T turbo pumped sputter coater (Quorum Technologies, Laughton, East Sussex, England BN8 6BN), and observed with a JEOL 7500F (field emission emitter) scanning electron microscope (JEOL LTd., Tokyo, Japan).

RESULTS

Previous research by our group analyzing the composition and development of microbial communities present in the tonsillar tissue of pigs using culture-independent sequencing of bacterial 16s rRNA genes demonstrated both the successional development of the tonsil microbiome and that the three most common taxa seen throughout the lives of pigs were *Streptococcus, Pasteurellaceae*, and *Moraxella* [21, 22, 29, 30]. Here we used both FISH to localize these three major taxa in tonsil tissue and SEM to characterize the overall structure of the tonsil tissue over the lives of pigs.

Validation of the probes. We validated our specific probes using fixed cells from pure cultures of the bacteria *Streptococcus suis*, *Staphylococcus aureus*, *Pasteurella multocida*, *Escherichia coli* and *Moraxella bovis*. Smears of mixed bacterial cells and the above mentioned combinations of probes were used to identify the positive labeling of cells with the morphological features of members of the genera *Streptococcus, Pasteurella* and *Moraxella,* when hybridized with their specific probe and when stained with DAPI, which stains nuclear DNA (Figure 4.1.A.). Bacterial cells inoculated onto muscle tissue showed similar results to those obtained with smears of mixed bacterial cells (Figure 4.1.B).

Spatial structure of communities on the tonsils using FISH. After validating the probes, tonsil samples were processed from all the 1, 3, 4 and 17 week piglets. FISH was performed with combinations of probes EUB338-NON338, EUB338-STR405-PAS11, MOR575-EUB338-PAS11 or MOR575-STR405-PAS11, all mounted with ProLong® Diamond antifade mountant with DAPI. There was positive labeling of *Streptococcus* cells with EUB338 or STR405 probes, and positive staining with DAPI in pig tonsil tissue; however there was no consistent specific labeling of tonsil specimens with either the PAS111 or MOR575 probes at any time point tested (Figure 4.1.C to 4.1.F). There was extensive non-specific labeling of granular structures disseminated on the epithelial surface (Figure 4.1.F and Figure 4.2.A.) as well as in other tissue locations (intraepithelial and in submucosa). The non-specific labeling was evident with all combinations of probes and made the definitive identification of positively labeled bacterial cells extremely difficult. An SEM image of the tonsillar surface from an eight week old pig showed some elements that could have been part of the

unspecific labeled material observed in FISH (Figure 4.2.B), and demonstrated all the material observed on the tonsillar surface that made the use of FISH difficult and problem prone.

Although previous research by our group [21, 22, 29, 30] using culture-independent sequencing of bacterial 16s rRNA genes showed that *Streptococcus*, *Pasteurellaceae*, and *Moraxellaceae* as well as many other taxa are present in pig tonsils, we were not able to reliably identify bacterial taxa other than members of genus *Streptococcus* or bacterial cells stained with the generic eubacterial probe using FISH. Therefore, we decided to use SEM to identify the presence and appearance of microbial communities in the tonsillar tissue of the sampled pigs.

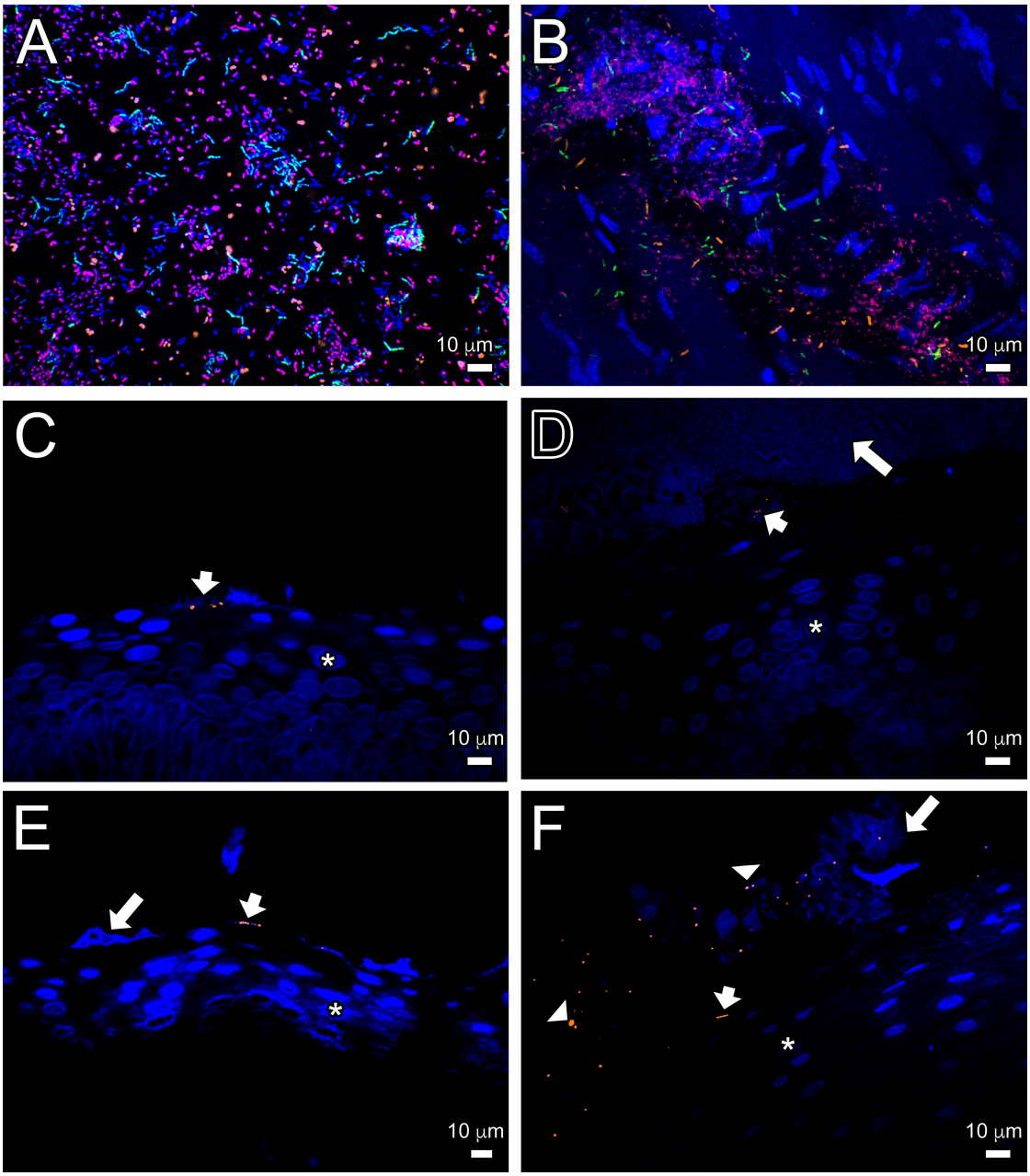

Figure 4.1. Representative images for the validation of FISH probes and identification of *Streptococcus* cells on the tonsillar surface. A, Oligonucleotide probes were validated with bacterial cell smears using DAPI staining (blue) and probes specific for *Moraxella* (green), *Streptococcus* (orange) and *Pasteurellaceae* (purple). B, Validated oligonucleotide probes using aseptically collected muscle inoculated with a mixed bacterial population. Nuclear DNA of myocytes (blue),

Figure 4.1. (cont´d). *Moraxella* (green), *Streptococcus* (orange) and *Pasteurellaceae* (purple). C, Tonsil tissue from a one week old pig. *Streptococcus* cells are visualized in orange (short arrow), lying on the surface of the nonkeratinized stratified squamous epithelia of tonsils (*). D, Tonsil tissue from a three week old pig. *Streptococcus* cells barely visualized in orange (short arrow), on the surface of the epithelium (*), covered by a layer of cellular debris (long arrow). E, Tonsil tissue from a four week old pig. Chain of *Streptococcus* (orange – short arrow) lying on the epithelial surface (*). Cellular debris (long arrow). F, Tonsil tissue from a ten week old pig. Long chain of *Streptococcus* (orange - short arrow) and cellular debris (long arrow), lying on the epithelial surface (*). Multiple structures with granular appearance and different sizes (arrow heads) are evident as well as cellular debris (long arrow) staining with DAPI (blue).

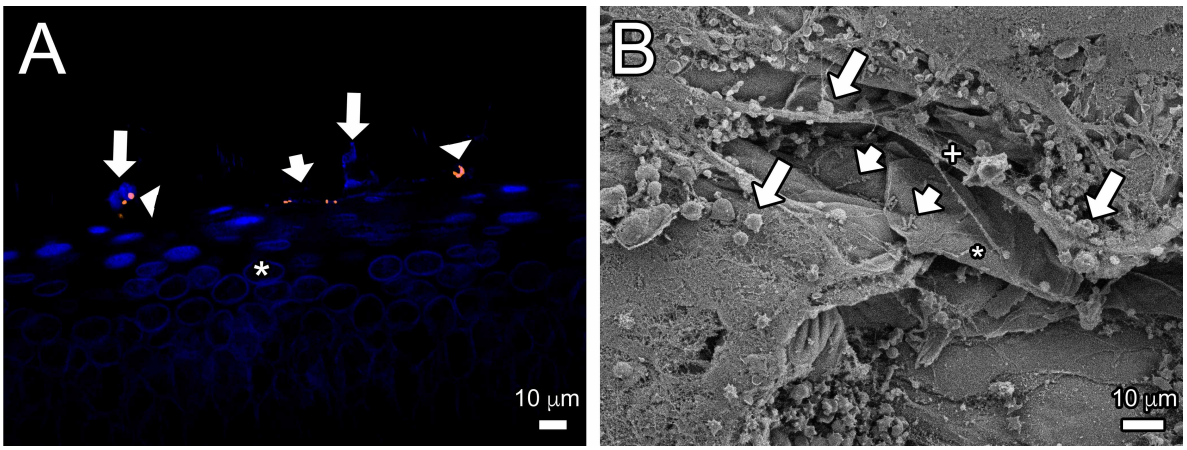

Figure 4.2. Representative images of bacterial cells and cellular debris in tonsils using FISH and SEM. A, Confocal image of tonsil tissue from a one week old pig showing positively hybridized *Streptococus* (orange - short arrow) on the epithelial surface (*) and structures of diverse sizes displaying non-specific labeling (orange –

Figure 4.2. (cont´d). arrow heads) and cellular debris (long arrows). B, SEM image from an eight week old pig showing bacterial cells (short arrows) on the tonsillar epithelial surface (*), surrounded by cellular debris (long arrows) and a layer of mucous/exopolysaccharide material (+).

Spatial structure of communities on the tonsils using SEM. Using SEM, tonsillar tissues of one, three, four, eight, ten and seventeen week old piglets showed a pattern of succession in the development of microbial communities in the tonsillar tissue of pigs. The characteristics of tonsils tended to vary between animals of the same age, with respect to the number of bacterial cells and amount of detritus identified. However, there were general features displayed by the pigs at each time point. The tonsillar surface of one week old pigs showed a smooth, clean surface with little debris (Figure 4.3.A), with crypts relatively clean and devoid of debris (Figure 4.3.B-4.3.C). There were scattered small communities of bacterial cells (Figure 4.3.D-4.3.E) composed mainly of lancet-shaped diplococci, long rods, short rods and diplobacilli. Also, two types of epithelial cell surfaces were evident: those with loosely arranged microplicae in microridges, and those with densely packed microplicae appearing as very short microvilli [31]. The initial attachment of bacterial cells to surface microplicae of the epithelial cells (Figure 4.3.F), using their pili, was clearly seen.

In three week old piglets, the tonsils were characterized by a tonsillar surface with a more rugose appearance (Figure 4.4.A) and wider crypt entrances. Increased debris was evident in the lumen of crypts (Figure 4.4.B to 4.4.D) and larger micro-communities of diverse bacteria were seen (Figure 4.4.C to 4.4.E). The random appearance of an echynocite was also observed (Figure 4.4.D). Bacterial micro-communities composed mainly of short rods, diplococci, diplobacilli and cocci were observed (Figure 4.4.E – 4.4.F).

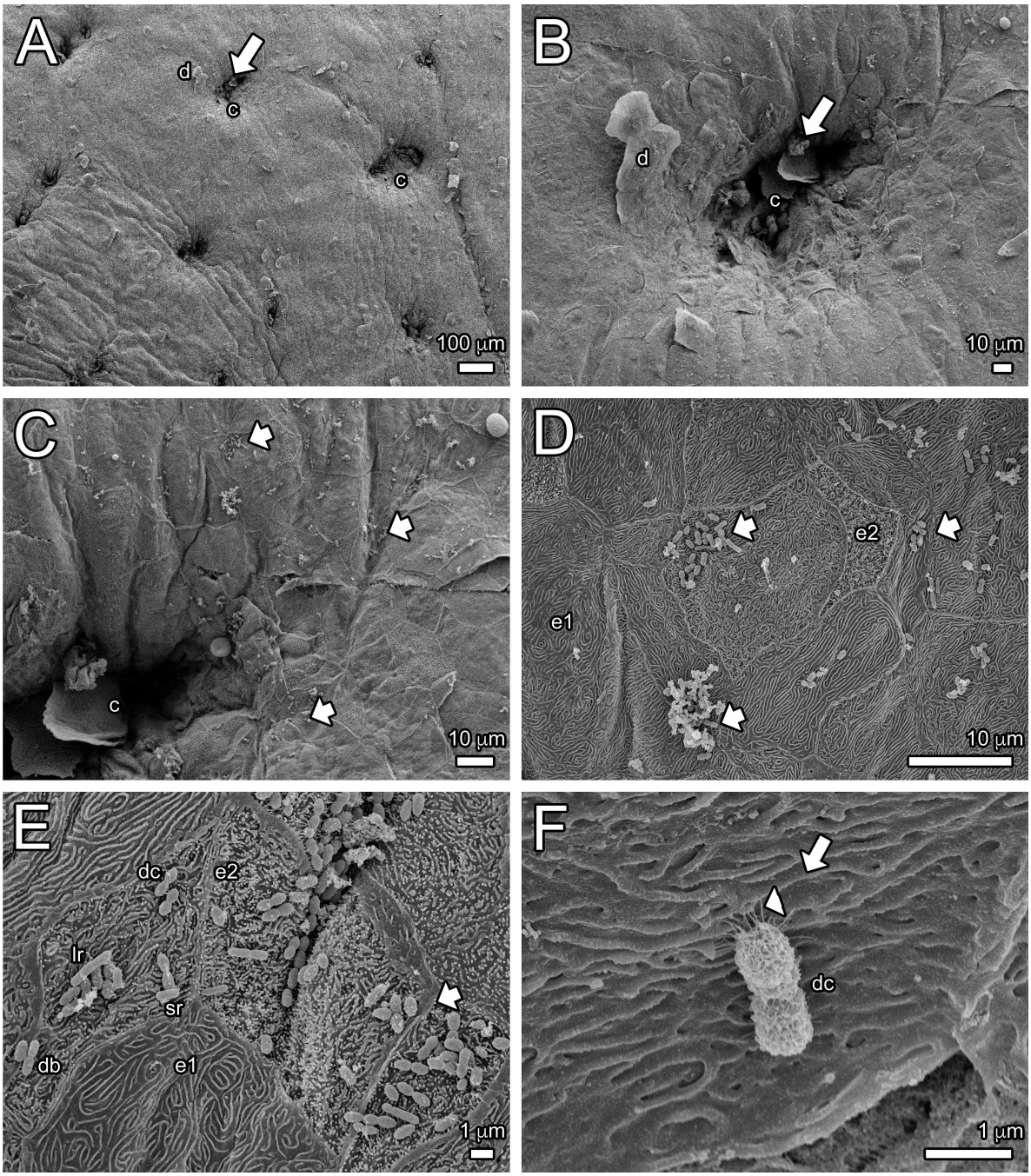

Figure 4.3. Representative scanning electron microscopy images of tonsil tissue from one week old pigs. A, tonsillar tissue of one week old pigs showing shallow crypts (c) with little debris (long arrow) and a very clean and smooth tonsil surface. B, Crypt (c) showing presence of a small amount of debris (long arrow) and desquamating epithelial cells (d). C, Demonstration of small sparse micro-colonies

Figure 4.3. (cont´d). (short arrow) around a crypt (c) entrance. D, Small micro-colonies of bacterial cells (short arrows) on the epithelial surface as well as few dispersed bacterial cells. Two types of epithelial cell surfaces with loosely arranged (e1) or densely packed (e2) microplicae are shown. E, Detailed view of a small micro-colony (short arrow) on epithelial cells showing the two types of epithelial cells with surface microplicae. Diverse bacterial morphologies: lancet-shaped diplococci (dc), long rods (lr), short rods (sr), diplobacillus (db), were present. F, Diplococci (dc) using pili (arrow head) to attach to the crests of microplicae (long arrow).

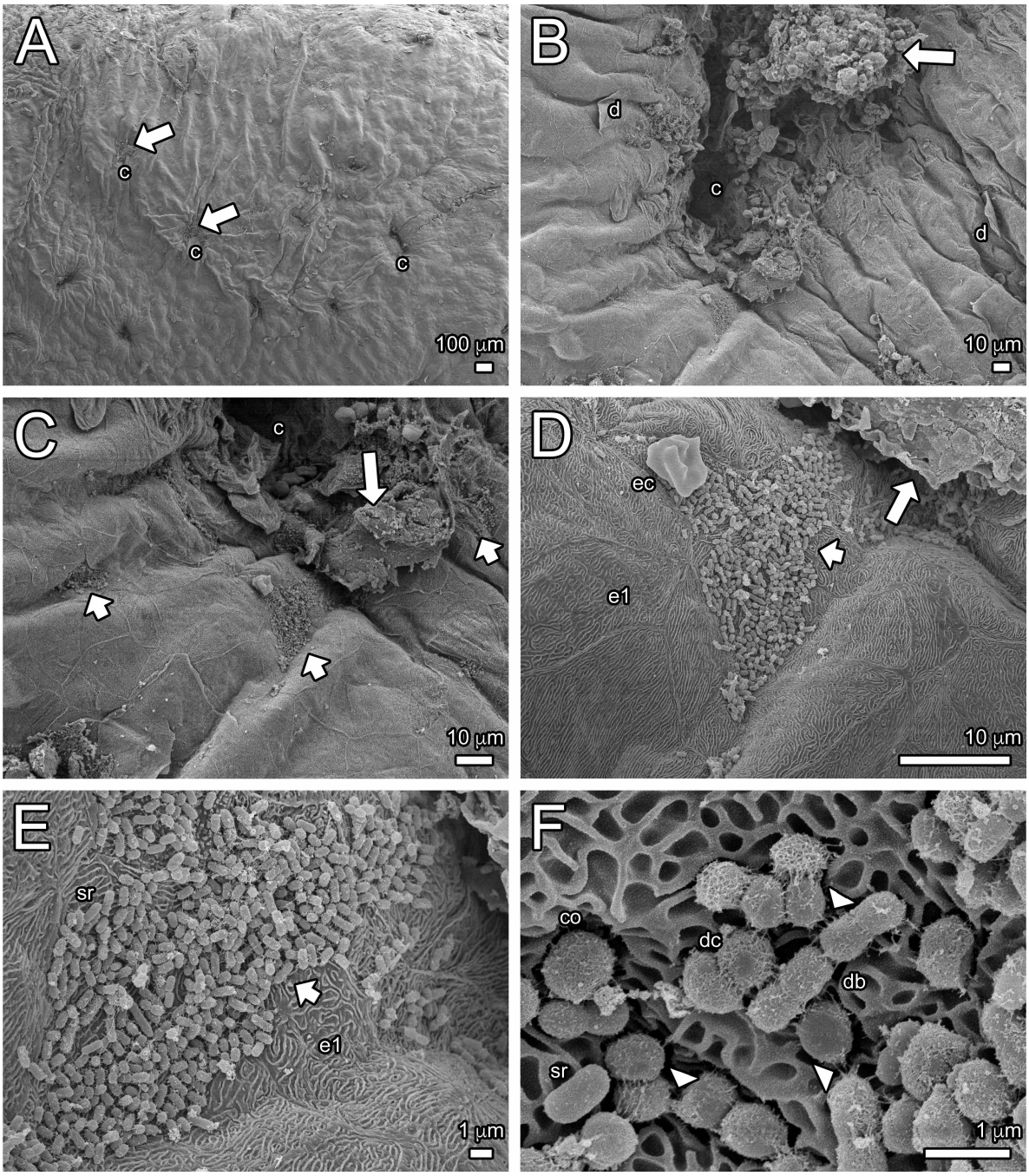

Figure 4.4. Representative scanning electron microscopy images of tonsil tissue from three week old pigs. A, tonsillar tissue showing the edges of crypts (c) becoming more rugose, and crypt entrance wider and with debris (long arrow). B, Lots of debris (long arrow) within crypts and some desquamating epithelial cells (d). C, Small clusters of bacteria micro-colonies (short arrow) scattered around a crypt

Figure 4.4. (cont´d). entrance. D, An echynocite (ec) lying beside a bacterial micro-colony on the epithelial surface. Bacterial cells are densely packed. E, Detailed view of a small micro-colony (short arrow) on an epithelial cell showing a population formed mainly by small rods (sr). F, Diverse bacterial morphologies: diplococci (dc), diplobacilli (db), cocci (co) and small rods (sr) interacting using their pili (arrow head).

The fourth week, which is marked as a stressful post weaning time with movement of the animals to new rooms, as well as switching to solid feed supplemented with an antibiotic growth promoter in the diet, showed a different view. Tonsillar crypt width continued to increase (Figure 4.5.A) and parts of the tonsillar surface were covered by patches and strands of debris and/or mucous/exopolysaccharide material (Figure 4.5.A to 4.5.E). The lumen of crypts appeared particularly empty. However, small micro-communities, mainly composed of diplococci and short rods and few large rods were identified around crypts (Figure 4.5.C to 4.5.F).

By the eighth week, after the piglets were on a solid diet supplemented with two different antibiotics used as growth promoters in feed for 4-5 weeks, the rugose appearance of the tonsillar surface returned and there was a noticeable amount of debris and mucous/exopolysaccharide material covering the lumen of crypts and the tonsillar surface (Figure 4.6.A – 4.6.B). Small clusters of micro-communities were evident in the periphery of the crypts (Figure 4.6.C). Long rods displaying a characteristic boxcar shaped morphology were widely seen (Figure 4.6.D – 4.6.E) as well as lower proportions of cocci, short rods and diplococci interacting and attaching to the cell surface and to the neighboring bacterial cells (Figure 4.6.E – 4.6.F).

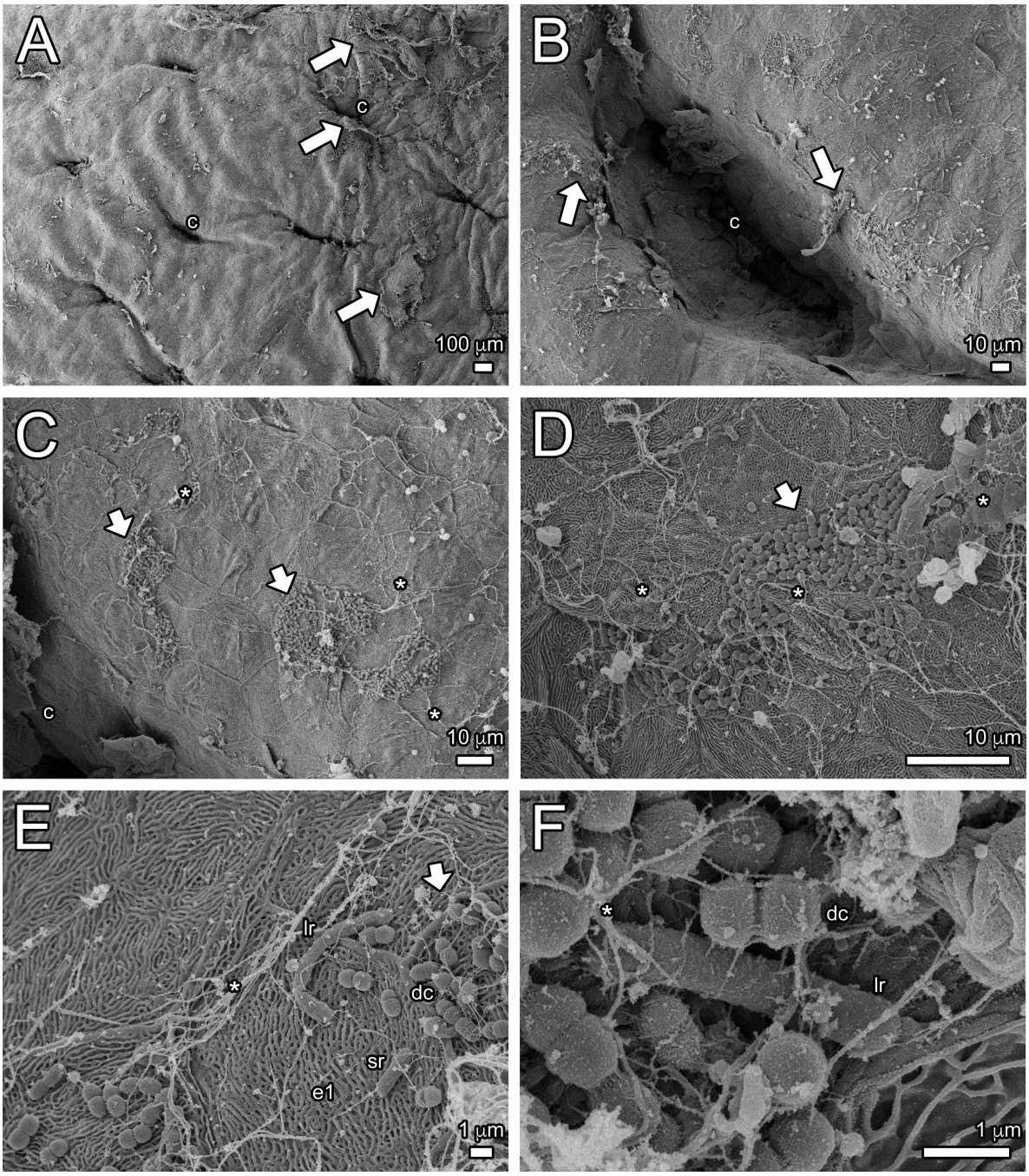

Figure 4.5. Representative scanning electron microscopy images of tonsil tissue from four week old pigs. A, tonsillar tissue of four week old pigs showing few patches of debris (long arrow) in the crypts (c), which had wider entrances, and covering the surface of tonsils. B, Crypt (c) almost empty, with very little debris (long arrow). C, Small clusters of bacterial micro-colonies (short arrow) scattered

Figure 4.5. (cont´d). around a crypt entrance (c), surrounded and/or covered with strands of mucous/exopolysaccharide material (*). D, Bacterial micro-colony (short arrow) on epithelial surface. Bacterial cells are densely packed and covered by strands and patches of mucous/exopolysaccharide material (*). E, Detailed view of a small micro-colony (short arrow) on an epithelial cell (e1) showing a relatively clear epithelial surface with presence of strands of mucous/exopolysaccharide material (*). Bacterial cells of the type diplococci (dc) as well as small rods (sr) and a large curved rod (lr) were evident. F, Lancet-shaped diplococci (dc) and long rods (lr) interacting and covered by strands of mucous/exopolysaccharide material (*).

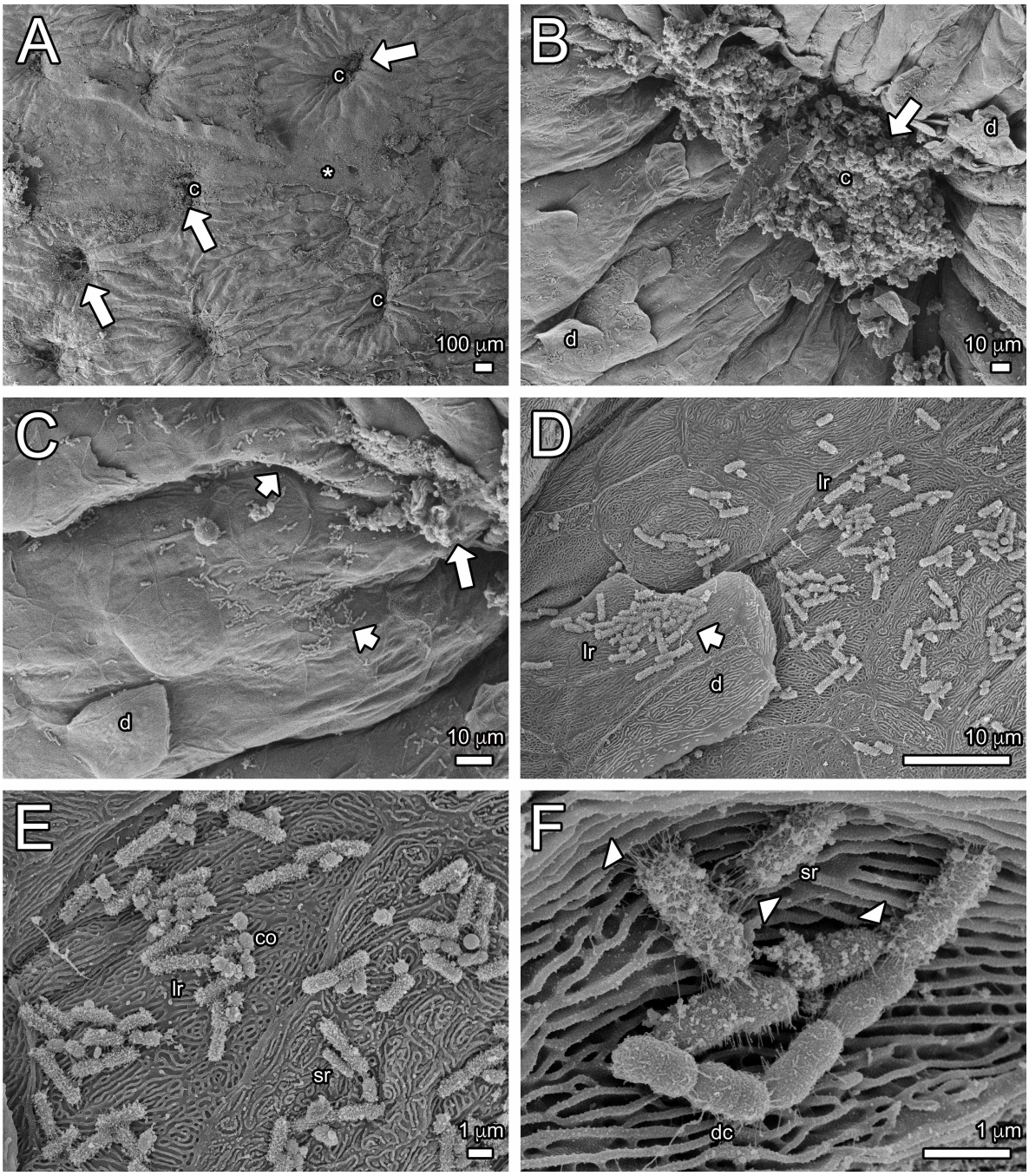

Figure 4.6. Representative scanning electron microscopy images of tonsil tissue from eight week old pigs. A, Tonsillar tissue showing rugose crypts (c) and a rugose appearance of the surface, with patches of scattered mucous/exopolysaccharide material (*) covering the surface of tonsils. B, Crypt (c) packed with abundant debris and some desquamating epithelial cells (d). C, Small clusters of bacterial

Figure 4.6. (cont´d). micro-colonies (arrow) scattered around a crypt, with some desquamating cells (d) and debris (long arrow). D, Bacterial micro-colony (short arrow) on the epithelial surface and on an apparently desquamating cell (d). Bacterial cell morphology was dominated by large boxcar-shaped rods (lr). E, Detailed view of a small micro-colony (short arrow) on an epithelial cell showing large boxcar-shaped rods (lr) intermingled with some cocci (co) and small rods (sr). F, Chain of diplococci (dc) and small rods (sr), using their pili (arrow head) to attach to the crests of microplicae and to the neighboring bacterial cells.

The panorama for the tenth week looked similar to week eight, where the tonsillar surface and crypts had a rugose appearance, crypts appeared wider, and the presence of debris and mucous/exopolysaccharide material was evident (Figure 4.7.A – 4.7.B). Broad micro-communities were observed in the periphery of the crypt lumen (Figure 4.7.C – 4.7.D), and were characterized by the presence of multiple cell morphologies: cocci, diplococci, and large and small rods, which were interacting closely via pili with each other and with the epithelial surface (Figures 4.7.E – 4.7.F).

Finally, tonsils in the seventeenth week displayed a very rugose tonsillar surface, extensively covered by a layer of mucous/exopolysaccharide material, with crypts that were very wide and full of debris (Figure 4.8.A – 4.8.B). There was considerable debris covering the epithelial surface (Figure 4.8.C), as well as extensive bacterial micro-communities (Figure 4.8.D), some of which were covered by a layer of mucous/exopolysaccharide material (Figure 4.8.E). Many bacterial cells appeared almost embedded in the surface of the tonsil epithelium. The bacterial cells were interconnecting extensively with the neighbor cells as depicted by the presence of multiple pili intermingling (Figure 4.8.F).

Similarly to the changes observed in the tonsillar surface of tonsils throughout the different samples, multiple bacterial members of micro-communities were identified through the different sampling times. These bacteria were characterized by diverse cellular morphologies, varying from lancet-shaped diplococci and small thin rods in the first week (Figure 4.9.A); large chains of palisade shaped *Alyssiella*, chains of cocci, short rods and long rods, in the third week (Figure 4.9.B); diplococci, long rods and short rods, in the fourth week (Figure 4.9.C); box-car shaped rods, lancet-

shaped diplococci, cocci and long rods, in the eighth week (Figure 4.9.D); long rods, cocci, lancet-shaped diplococci and small rods, in the tenth week (Figure 4.9.E); and finally, cocci and small rods in the seventeenth week (Figure 4.9.F).

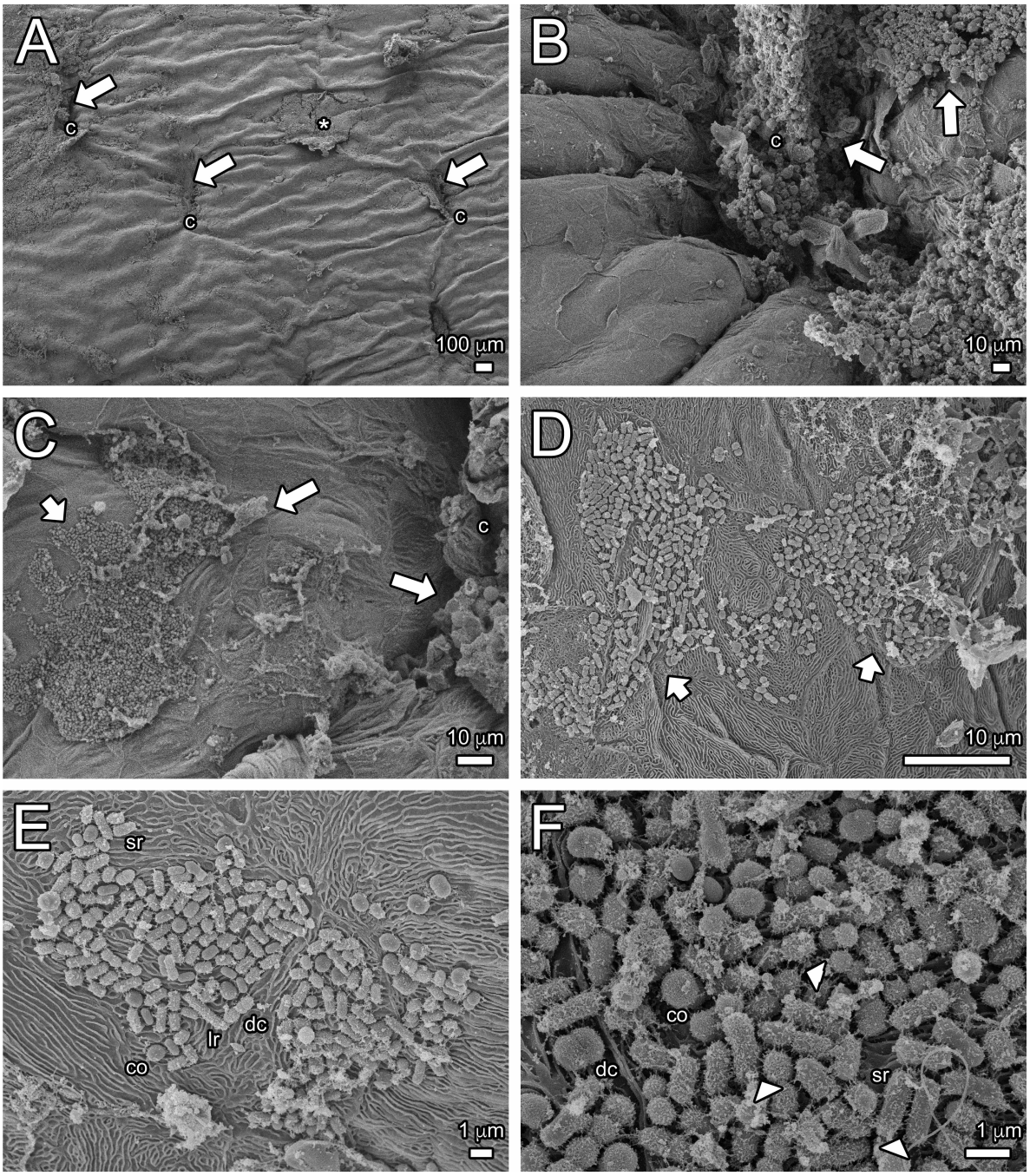

Figure 4.7. Representative scanning electron microscopy images of tonsil tissue from ten week old pigs. A, Tonsillar tissue showing rugose crypts (c) and a rugose appearance of the surface. Abundant debris (long arrow) in the crypt entrance and mucous/exopolysaccharide material (*) covering patches of the tonsil surface. B, Crypt (c) with abundant debris (long arrow) in the lumen and covering the walls.

Figure 4.7. (cont´d). C, Large bacterial micro-colony (short arrow) in the entrance of a crypt (c), surrounded by scattered debris (long arrow). D, Bacterial micro-colonies (short arrows) with diverse clustered cells covering large areas of the epithelial surface. E, Detailed view of a bacterial micro-colony showing an intermingled population of cocci (co), large rods (lr), small rods (sr) and diplococci (dc). F, A diverse bacterial community showing interactions between small rods (sr), cocci (co), diplococci (dc) extending their pili (arrow head) to the neighboring cells.

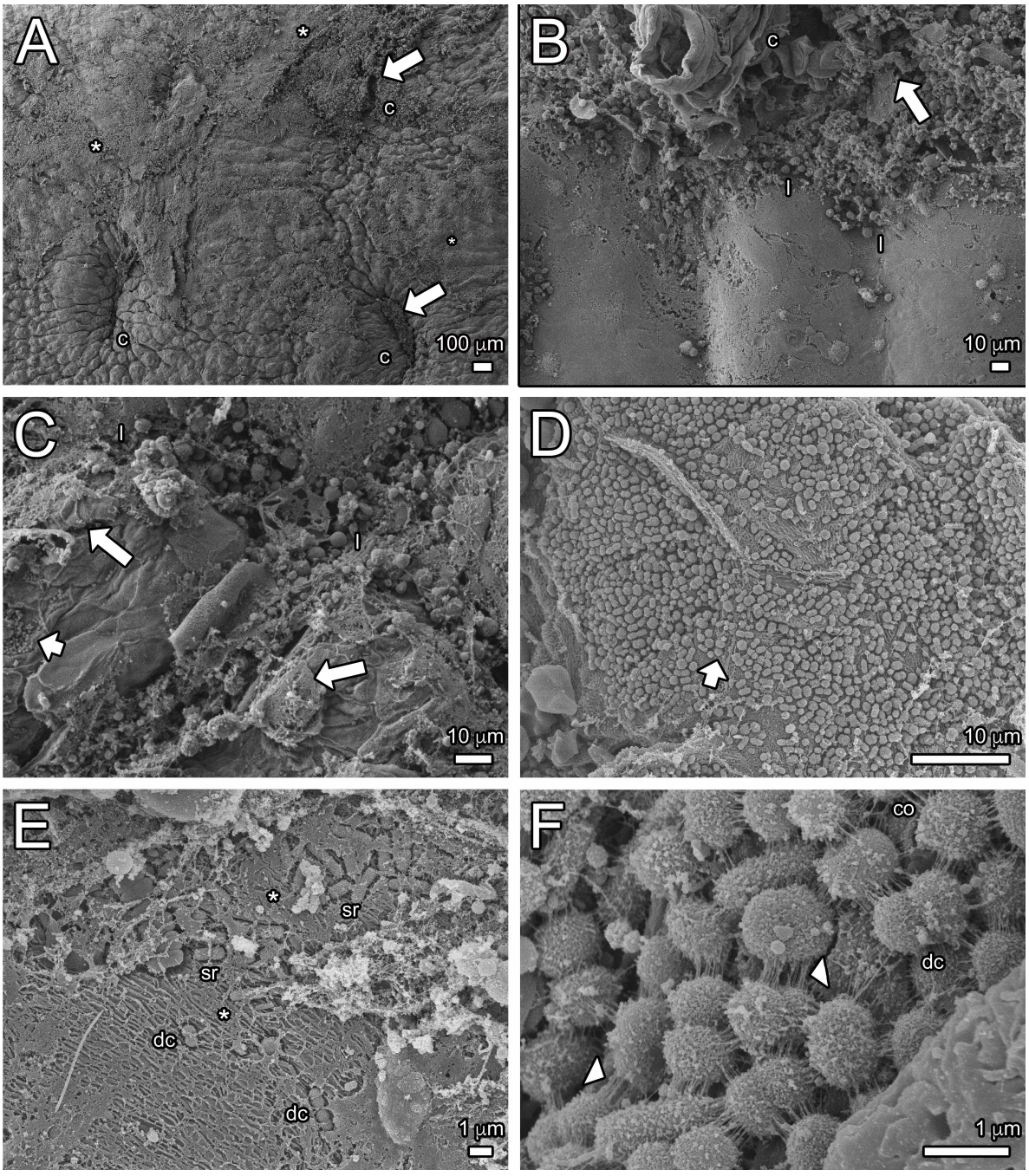

Figure 4.8. Representative scanning electron microscopy images of tonsil tissue from seventeen week old pigs. A, Tonsillar surface extensively covered by a layer of mucous/exopolysaccharide material (*) and crypts (c) full of debris (long arrow). The epithelial surface has a very rugose appearance. B, Crypt fully clogged with debris (long arrow). Clear presence of round structures with smooth surface, likely

Figure 4.8. (cont´d). lymphocytes (l). C, Epithelial surface extensively covered with debris (long arrow). A bacterial micro-colony (short arrow) and multiple round and smooth structures, likely lymphocytes (l) are present. D, Epithelial surface extensively covered by a bacterial micro-colony (short arrow). E, Detailed view of diplococci (dc) and small rods (sr), appearing almost embedded in the tonsil epithelial surface and covered by a layer of mucous/exopolysaccharide material (*). F, Diplococci (dc) and cocci (co) interacting extensively, with pili (arrow head) extended to the neighboring cells.

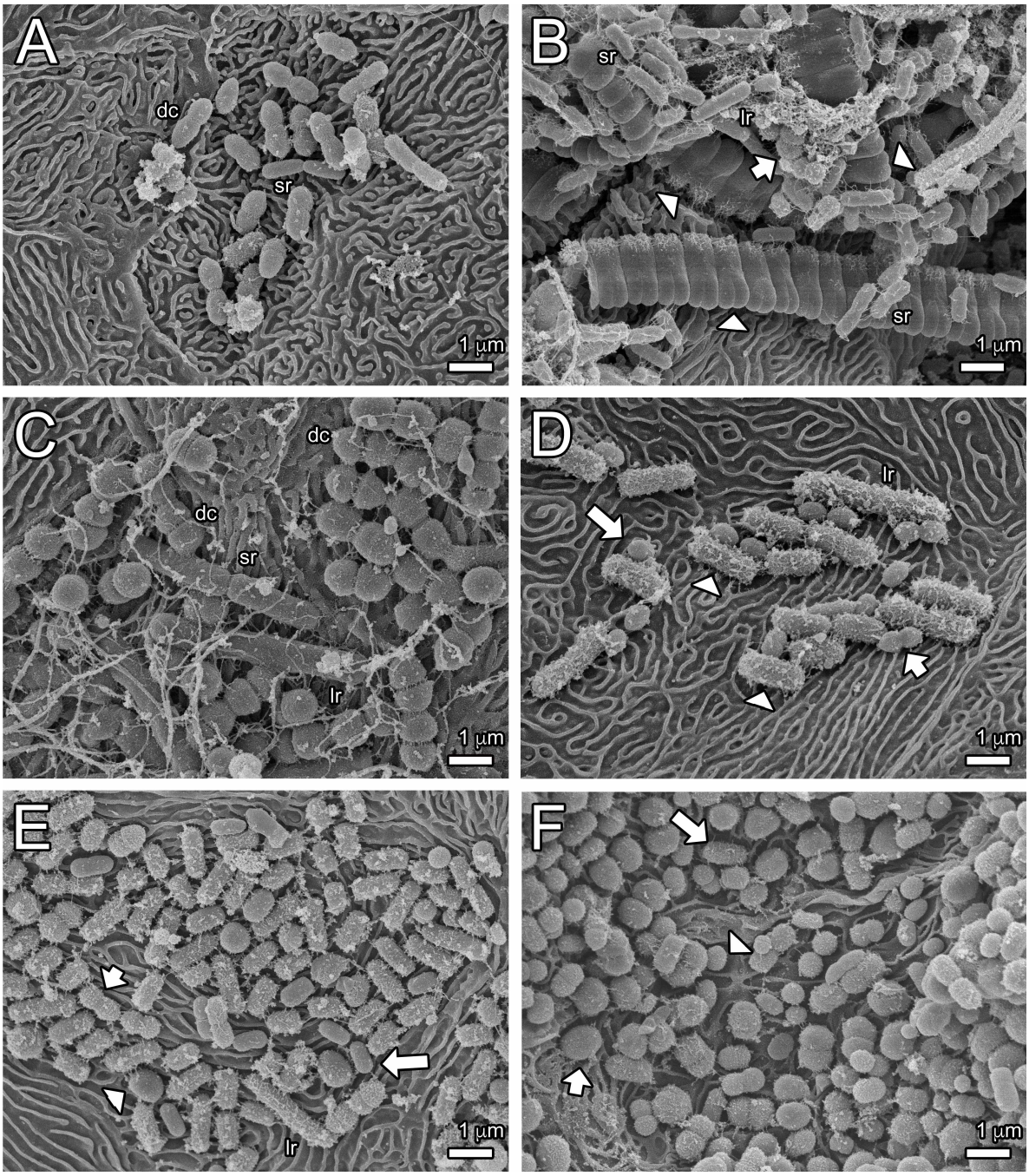

Figure 4.9. Representative bacterial micro-colonies identified through the different sampling times. Scanning electron microscopy images from tonsil samples of pigs in different times of their life. A, Micro-colony formed by lancet-shaped diplococci (dc) and small thin rods (sr), identified in one week old pigs. Bacterial cells are loosely disseminated on the epithelial surface. B, Micro-colony composed of

Figure 4.9. (cont´d). several large palisade-shaped chains of actively dividing bacteria with pili along one short end of each cell, which are likely *Alyssiella* [32](arrow head), interacting with a chain of cocci (short arrow), short rods (sr) and long rods (large arrow), identified in a three week old pig. C, Micro-colony composed of diplococci (dc), long rods (lr), and short rods (sr), identified in a four week old pig. D, Micro-colony composed of box-car shaped rods (arrow head), lancet-shaped diplococci (short arrow), cocci (long arrow) and long rods (lr), identified in an eight week old pig. E. Micro-colony composed of long rods (lr), cocci (arrow head), lancet-shaped diplococci (short arrow), small rods (long arrow), identified in a ten week old pig. Extensive interactions between bacteria. F. Micro-colony composed of cocci (short arrow) and small rods (large arrow), closely interacting. Bacterial cells seem embedded in the surface of the epithelial cells.

DISCUSSION

The present study allowed us to investigate the temporal succession of tonsillar bacterial community structures in pigs, from one week through seventeen weeks old. The validation of the oligonucleotide probes with smears of bacterial cells as wells as with muscle inoculated with bacterial cells was successful. However, when the FISH probes were used on tissues derived from pig tonsils, only bacteria from genus *Streptococcus* were reliably identified on the epithelial surface of tonsils from the sampled pigs. While *Streptococcus* was not present in every slide for each animal, there was positive identification of *Streptococcus* in at least one sample examined for each sampling time. The initial aim of this study was to characterize the assembly of the three most abundant taxa identified - *Pasteurellaceae*, *Streptococcus* and *Moraxella* - through the development of the tonsillar microbiome of pigs, which have been reported as being present throughout the lives of the pigs from this farm [21, 22]. Nevertheless, the presence of marked nonspecific staining of multiple structures (Figure 4.1.F and 4.2.A) in most of the samples with all the fluorophores used, especially Cy3 and Cy5, as well as the marked autofluorescence of the tonsil tissue, made this aim unattainable in the given circumstances. The issues of non-specific background and the possible biases when analyzing eukaryotic tissues processed by FISH have been reported previously [33]. Further, tissue autofluorescence has been associated with a decrease in the signal-to-noise ratio and with masking the true fluorescent signal, when working with FISH [34]. We did not quantify these phenomena in our study, but certainly the high presence of non-specific background

and autofluorescence negatively influenced our ability to detect reliably the signal of the hybridized probes.

It is difficult to detect bacteria when using FISH probes in eukaryotic tissue when the bacterial concentration is lower than 10^5/ml [35]. Hogart et al [36] demonstrated that FISH is a highly specific technique, albeit with a moderate sensitivity when dealing with bacteria with concentrations lower than 4 x 10^5 CFU/ml in sputum samples. Stepinska et al [37] compared the use of culture isolation vs FISH techniques for the identification of bacterial cells from lysates of CD14 cells from adenoids and tonsil tissue, and although they stated that FISH was twice more effective than cultivation in detecting bacteria, they also could not obtain positive FISH results in some of the samples, in spite of being able to isolate bacterial cells from them. We do not know the exact concentration of bacterial cells in each of our samples, although we have previously found 1 X 10^6 – 1.5 X 10^7 cfu per gram of tonsil tissue in healthy grower-finisher pigs. Certainly, using a culture-independent approach by sequencing the 16s rRNA genes of tonsillar samples collected from pigs from the same farm, we characterized these three bacterial families (*Pasteurellaceae*, *Streptococcaceae* and *Moraxellaceae*) as present in a high relative abundance through the pigs' life [21, 22].

Moter and Göbel [34] have described the possible problems and pitfalls in using FISH for visualization of microorganisms. The authors mentioned autofluorescence and lack of specificity of nucleotide probes as possible causes of false positive results in FISH procedures. Additionally, they mentioned the insufficient penetration of the probes, higher order structure of target/probe, low RNA content, as

well as photobleaching and failure to use a probe targeting bacteria in general, as a cause of false negative results. In the present study, it is possible that the non-detection of the expected microorganisms in the samples may be due to our strict quality control, where we did not accept what we considered to be possible false positive results where we saw tissue autofluorescence or non-specific labeling of eukaryotic cells or granules with our probes. Since the oligonucleotide probes were validated with bacterial isolates from the desired taxa, we considered that we were targeting the specific desired microorganisms. Probes STR405, PAS111 and EUB338 were validated under our experimental conditions. These probes have been validated in previous studies of microbial communities in the oral cavity of humans [16]. The aforementioned probes targeted a very broad spectrum of microorganisms in each of the desired taxa. We also validated them *in silico* against representative sequences for the taxa identified as more abundant in samples from the sampled farm (data not shown). We were expecting positive identification of members of bacterial taxa related with the probes. On the other hand, we designed a probe targeting a broad spectrum of microorganisms of the genus *Moraxella* (MOR831), that was tested *in silico* as a good probe however, we were not able to validate the probe against multiple *Moraxella* isolates (data not shown). After analyzing our designed probe, we found that it was targeting a 16s rRNA location characterized by difficult accessibility and giving a very poor fluorescence [38]. We designed a second probe (MOR575) targeting members of genus *Moraxella*. Unfortunately, the designed probe only targeted a narrow group of microorganisms in this genus; however we validated the probe *in silico* against representative sequences of this taxa identified in our previous studies

[21, 22], as well as against diverse *Moraxella* isolates stored in our lab collection of microorganisms. We did not culture *Moraxella* isolates from the sampled pigs, which would have been the ideal to validate our probe with even more specificity. The designed probe when examined, still provided a relative low fluorescence intensity (40%) as mentioned by Fush et al [38]. It may be possible that the inability to identify microorganisms from the genus *Moraxella* in the samples, was due to the excessive autofluorescence of the tissue samples and the low signal-to-noise ratio of the probe. It also may be due to the fact that the microorganisms from this genus present in the samples were not targeted specifically by the probe.

Swidsinski and Loening-Baucke [39], reported the almost total absence of bacteria in the epithelial surface of human tonsils, even in samples derived from tonsillectomy of patients with chronic tonsillitis. However, the positive identification of bacteria by FISH, in tonsils derived from patients with chronic tonsillitis has been reported [35, 39]. The authors identified high concentrations of bacteria attached to tonsils/adenoids epithelia, bacterial infiltrations of superficial epithelia, bacteria in tissue fistulas and/or fissures, as well as bacteria contained in macrophages, in regions of tissue associated with an inflammatory response. In the present study, it may be possible that what we sometimes identified as non-specific labeling of granular structures in the submucosa may in fact be positive hybridization to bacterial cells inside or attached to phagocytic cells, masked by the autofluorescence and non-specific labeling of other tissue components that we observed. Further, the large amount of mucous/exopolysaccharide often seen on the tonsil surface and within the

crypts, as well as the layers of bacteria seen in colonies in the older piglets, may have blocked access of the probes to many bacteria.

Despite the negative results attained in this study to investigate the temporal succession of tonsillar bacterial community structures in pigs by using FISH, when we used SEM, we were able to identify a pattern of succession in the appearance of the tonsillar surface as well as in the width of the crypts and more importantly, in the structure of the tonsillar micro-communities observed. Initially for newborn pigs, the tonsillar surface had a smooth and relatively clean surface but as pigs aged, the tonsillar surface acquired a more rugose texture. The crypt entrances also changed as pigs aged, with the width of the crypts on average increasing and the crypts being filled with debris including mucous/exopolysaccharide material, lymphocytes and cellular detritus, among other material. The presence of leucocytes, Periodic acid-Shiff (PAS) positive content and bacteria in the lumen of crypts has been reported [40].

Samples from one week old pigs were characterized by small micro-communities composed mainly of lancet-shaped diplococci and small rods. We identified the clear attachment of many of these diplococci to the crest of microplicae in the epithelial surface of tonsils. It has been suggested that the crest of epithelial microplicae are used for initial attachment of bacteria [41] and this was demonstrated for *Streptococcus pyogenes* [42]. Certainly, the number of members of the identified micro-communities increased with the time as well as the interactions between individual cells with the neighboring cells. We observed micro-colonies with bacterial cells apparently embedded in the epithelial surface. Similar findings of bacteria forming apparent depressions in the cell surface of epithelial cells in tonsils have been

reported [41]. We saw a greater number of cells connected via pili to neighboring cells as the pigs aged. The microbial communities identified for seventeen weeks old pigs seemed to be covered by a layer of mucous/exopolysaccharide material. It was not an aim of this study to classify the chemical nature of this material. However, Kania et al [43], with the use of SEM, FISH and special stains, demonstrated the presence of bacterial micro-colonies immersed in a matrix of glycocalix in tonsils from children with chronic or recurrent tonsilitis, which they called biofilms. We only can say that the structure of the micro-communities or micro-colonies changed with time as more cells were included in the micro-community. Similarly, the interactions of the members of those micro-communities seemed to increase with the age of the pigs, as we observed more cells interacting via pili with the neighboring cells in the micro-community. We also saw large numbers of lymphocytes and red blood cells, as well as echynocytes (deformed red blood cells) in many of the samples.

Although unfortunately, we cannot classify the bacterial cells observed with SEM taxonomically beyond the cell morphology, we certainly saw different bacterial shapes forming the micro-communities throughout the life of the pigs. We have been able to isolate *Streptococcus suis* in many piglets from this farm at birth, and from all the piglets from the same litter by one day of age (unpublished data). We studied the development of the tonsillar microbiome in pigs from the farm and we identified different OTUs assigned to *Streptococcus*, but particularly *Streptococcus suis* in at birth piglets as well as constantly throughout the life of pigs [21, 22]. It has been demonstrated that in the formation of dental plaque in humans, *Streptococcus* acts as a first colonizer and facilitates the attachment/aggregation of other members of the

bacterial community [7]. This pattern of adhesions and interactions between mixed species forms the basis of spatiotemporal development of the dental plaque [5]. We can only speculate that the diplococcus-shape cells that we saw almost constantly in all the samples may be a member of genus *Streptococcus* acting as first colonizer and facilitating the building up of the bacterial micro-communities observed in all our samples. Members of genus *Streptococcus* were positively identified in samples from these pigs, using FISH probes targeting members of this genus. It is possible that bacteria from genus *Streptococcus* are facilitating the attachment of bacterial cells from other taxa (different bacterial shapes observed) and building up the communities identified.

In conclusion, we used FISH and SEM to identify the structure and presence of diverse bacterial communities on the surface of pig tonsils through different times in their life. We identified morphological changes in the appearance of pig tonsils and the bacterial members of the communities on tonsils from one, three, four, eight, ten and seventeen weeks old. To the best of our knowledge, this is the first published study describing the morphological changes of the tonsillar surface in pigs associated with changes in the microbial communities observed through different times in their life.

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CHAPTER 5. SUMMARY AND FUTURE DIRECTIONS

SUMMARY

Tonsils are recognized as one of the first tissues that can be colonized by pathogens entering the respiratory or oral pathway. Tonsils can be used as a primary site of replication for some bacterial and viral agents that can be either host-specific or zoonotic organisms. Some of these bacterial agents, such as *Streptococcus suis,* are isolated continuously from pig tonsils and deserve special attention especially because of its zoonotic potential and economic impact. Further, it is recognized that the tissue-resident bacterial microbiome may play an important role in host protection against colonization by pathogenic microorganisms. The goal of this study was to characterize the development of the composition and structure of the tonsillar microbiome in pigs from birth through market age, thus laying the foundation for future studies concerning the interaction of pathogens with resident microbial communities in the tonsils.

Studies of the tonsillar microbiome of pigs, using a culture-independent approach based on the use of cloning libraries, have identified that in 18 to 20 week old pigs from two different healthy herds, ~74% of the microbiome was composed of members of the families *Pasteurellaceae*, *Porphyromonadaceae*, *Bacteroidaceae* and *Prevotellaceae* [1]. However, using a 454-pyrosequencing approach to study the same samples and new samples from the same farm, it was found that ~ 90.4% of the microbiome was composed by members of families *Pasteurellaceae*, *Moraxellaceae*, *Fusobacteriaceae*, *Veillonellaceae*, *Neisseriaceae*, *Peptostreptococcaceae*, *Enterobacteriaceae* and *Streptococcaceae* [2]. Notwhitstanding these previous reports studying the microbiome in pig tonsils, there are no studies focused on characterizing the development of the tonsil microbiome in pigs.

Based on studies conducted in pigs and humans, it has been suggested that there is a gradual and successional process in the development of the intestinal microbiome [3-6]. We hypothesized that the development of tonsillar microbial communities in pigs also follows a temporal succession process. Since there are no published reports characterizing the development of microbial communities in pig tonsils, we decided to study and understand the development of the microbiome in pig tonsils, in continuance of the previous work done in this field by our laboratory.

Our lab has previously developed and validated a non-invasive method to collect tonsil samples and used this method to describe the core tonsil microbiome in grower-finisher pigs. The use of this validated methodology allowed us to follow the microbiome development of each animal continuously from birth (day 0) up to market age (17 weeks). Our initial approach was to analyze the development of tonsillar microbiome in pigs during the first few weeks of age, giving special attention to the possible sources of the members of the tonsil community and to the challenging time of weaning and the effects of post weaning in the tonsillar microbiome communities. We found that the microbiome in piglets, collected a few hour post birth (PB), showed organisms including *Streptococcus*, *Staphylococcus, Moraxella, Rothia,* and *Pasteurellaceae* (OTUs 002, 009, 003, 007, and 001 respectively) as the most abundant in tonsils at that time. We compared these with OTUs found in maternal vaginal, teat skin, tonsil and fecal samples and traced that, piglets acquired *Pasteurellaceae* and *Streptococcus* from the sow vaginal tract most likely during parturition, while sow teat skin (or milk, which we did not sample) was the likely source for *Moraxella, Staphylococcus* and *Rothia*. These results are in agreement with studies of Mandar and Mikelsaar [5], which compared vaginal flora of human mothers with the

microorganisms that initially colonize the external ear canal in their newborns and concluded that there is a significant influence of the vaginal microflora in the initial microbial population found in the newborns. *Streptococcus* and *Staphylococcus* as well as other aerobes have been commonly reported as first colonizers in infants, possibly derived from maternal sources, such as breast milk and vagina.

Our results also demonstrated a strong litter effect in the initial development of tonsillar microbiome in pigs, followed by a gradual successional development, where, by the third week, the microbiomes of all piglets was highly similar. The piglet tonsil microbiomes were clustered tightly by litter at birth and at week 1. However, this clustering was no longer apparent by 3 weeks of age. In the successional development, some organisms found in high proportions initially in piglets PB, such as *Staphylococcus* and *Micrococcaceae*, decreased dramatically within the next 2 weeks. However, members of the families *Pasteurellaceae* and *Moraxellaceae*, increased their levels in parallel, while the levels of others decreased mild to moderate, as the case of *Streptococcaceae*. Furthermore, there were also several transitory OTUs or families that appeared and disappeared at specific time points such as *Porphyromonadaceae, Prevotellaceae,* and *Flavobacteriaceae* that appeared at week 1 and increased slightly over the next 2 weeks or such as *Fusobacteriaceae* and *Leptotrichiaceae,* that appeared for two weeks and then almost disappeared. Similar results in the succession and shifts in the population assembly which seemed to stabilize over time, were reported in the development of intestinal microbiome in humans and pigs [5, 6].

Our work has demonstrated that weaning and the significant stress events associated with it, such as environmental, social and feed changes, were associated with a

major shift in the microbiome. These results are in concordance with studies of development of intestinal microbiome which demonstrated that transition from nursery to weaning is associated with a significant change in the intestinal microbiota [8, 9].

After having identified that there is a succession in the microbiome in the first weeks of life and that weaning generated significant shifts in the microbiome, we followed how the microbiome develops further during the life of pigs, particularly where they are exposed to different challenges associated with normal management practices in a swine farm. We identified four major challenging conditions that were related with perceptible changes in the relative abundance of the tonsillar microbiome populations. The first challenge was previously described as weaning, which occurred in parallel with the vaccination of piglets, movement to a nursery room, and introduction to a solid diet based on pellet ration supplemented with Carbadox®. The second challenge was associated with withdrawal of Carbadox® supplementation and a shift to feeding with a ground ration supplemented with Tylan®. The third challenge was associated with the move to a finishing room, as well as the separation from litter mates and discontinuation of Tylan® supplementation from the ground ration. The final identified challenge was associated with another move to the final finishing room.

The identified challenges were associated with shifts in the composition and relative percentages of families of the microbiota. These challenges frequently occurred as combinations of several stress and rarely involved a singular change in the management of the pigs. Nevertheless, supplementation with Carbadox® or Tylan® have been associated with shifts in the distribution of microbial communities in the intestine [10, 11]. Similarly, changes in the environment where pigs grow [12, 13], and changes in their diet [8, 14, 15],

have been associated also with shifts in the microbial communities in intestine. However, there are not specific studies that refer to changes experienced by microbial communities of pig tonsils when the aforementioned conditions are present.

The study of microbial communities in tonsils from newborn to market age confirmed that there is a succession in the development of the communities through different ages of the pigs. However, there were some bacterial families that dominated the tonsillar microbiome through-out the life of pigs such as members of *Pasteurellaceae*, *Streptococcaceae* and *Moraxellaceae*, although their relative abundance experienced changes after the challenging events. These findings are supported by studies done in fecal microbiome in pigs, reporting that some phyla dominate the microbiome regardless of the age [16]. On other hand, we found that older pigs (16 weeks) tended to have a microbiome more similar to the microbiome of older animals, for example sows. Similarly, studies in human intestinal microbiota have shown that as infants age, their microbiota was more similar to adults microbiota [3, 5].

Finally, using FISH and SEM, we confirmed the presence of diverse bacterial communities on the surface of pig tonsils and how these changed through their life. Further, we confirmed the presence of members of genus *Streptococcus* on tonsillar surface of pigs. Finally, we have also identified morphological changes in the appearance of pig tonsils and the bacterial members of the tonsil communities throughout the life of pigs.

FUTURE DIRECTIONS

The results of this study demonstrated that there is a succession in the development of tonsillar communities in pigs. We identified the presence of some bacterial families all through the life of pigs while others fluctuate or appeared and disappeared sporadically. We can only speculate on the initial sources of tonsillar microbiome. We don´t know the effect of the use of Carbadox® and Tylan® or other antibiotics in the development and succession of the tonsillar communities. More importantly, we don´t know the role played by normal microbiota in the acquisition of a pathogenic microorganism. We may have also ignored the exact role played by some members of the microbial communities in the development of microbiome structure and in the acquisition of potential pathogens. Finally, we do not know if the arrival of these pathogens can be prevented by the use of probiotics or specific changes in the management practices. All these questions will be addressed in future works however; initially we want to address the following topics:

Future direction 1. Follow the development of the tonsil microbiome in a second herd, possibly one with a chronic problem such as *Salmonella*.

Future direction 2. Examine effect of acquisition of a pathogen known to colonize the tonsils, such as *Actinobacilllus pleuropneumoniae* or *Salmonella enterica*, on the composition of the tonsil microbiome.

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