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DEVELOPMENT OF A PORCINE LACTATION MODEL FOR THE EVALUATION OF MAMMARY TRANSFER OF EXOGENOUS MOLECULES A CONTRIBUTION FROM THE CONCEPTION PROJECT

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Abstract

More than 5 million women give birth each year in Europe and, while breastfeeding, the majority of them may need to take medications, either occasionally or continuously. Unfortunately, there is often scarce evidence of trustworthy information about how a specific molecule might affect the physiology of lactation and the nursing child. This is the reason that brought a European public-private partnership to fund the development of a reliable platform, capable of providing women and health-care professionals a helpful instrument to reduce uncertainty about the effects of medication used during breastfeeding. On April 1st 2019, the ConcePTION project (Grant Agreement n°821520) started aiming to develop such envisaged platform. Within the eight Work Packages of ConcePTION, the 3rd is in charge of the validation of *in vitro*, *in vivo* and *in silico* lactation models able to predict drug concentration in human breast milk. Between the numerous species currently used in preclinical studies, pigs' similarities with humans' anatomy, physiology and genomics make them extremely useful as translational models, when proper veterinary expertise is applied. The ASA team from the Department of Veterinary Medical Sciences, University of Bologna, being part of ConcePTION project, went first to characterize the translational lactation model using the swine species, chosen upon literature review.

The aim of this work was to lay the foundations of a porcine lactation model that could be suitable for application within industrial pharmaceutical tests, to study drug transfer through milk prior approval and commercialization. The obtained results highlighted both strengths and critical points of the study design, allowing a significant improvement in the knowledge of pharmacokinetic physiology in lactating mammals. Lastly, this project allowed the assessment of microbial changes in gut resident bacteria of newborns through an innovative *in vitro* colonic model. Indeed, even if there were no evident adverse effects determined by drug residues in milk, possible alterations in the delicate microbial ecology of newborns' gastrointestinal tract was considered pivotal, giving its possible impact on the individual health and growth.

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Introduction

1. Animals in Biomedical Research

1.1 History of Animal Modeling

The expression “Animal Experimentation” refers to the scientific study of animals for the acquisition of new biological knowledge or to solve specific biomedical issues. Nowadays, most animals employed in experimentations are bred and housed in laboratories under standardized environmental conditions (Fox 2015).

The first evidences of animal experiments trace back to the works of Greek philosopher and physician Aristotle (384 – 322 BC), generally acknowledged as the founder of biology, and his compatriot Erasistratus (304 – 250 BC), an anatomist, most likely the first to perform experiments on living animals (Ericsson et al. 2013). Their early instances of comparative science had an observational feature, aiming at better understanding mammals’ anatomy and physiology. Afterwards, Galen (130 – 200 AD) reported anatomical studies based mainly on the dissection of apes monkeys, pigs and many other species; he is considered to be one of the most accomplished medical researchers of the Roman Empire (Hankinson 2008).

Despite dissection of human and animal cadavers being among the earliest examples of experimentation, such practice was banned amid Middle Ages by ecclesiastical authorities, who considered many scientific disciplines as blasphemous (Ferngren 2017). The sixteenth century saw a marked reawakening of the interest in science; the famous anatomist Andreas Vesalius (1514 – 1564 AD) used to perform anatomical demonstrations in public, using vivisection on dogs and pigs (Fox 2015). Vesalius’ experiments, as cruel as they might have been, allowed him to understand the intimate mutual correspondence between anatomy and physiology. Later on, William Harvey (1578 – 1657 AD) assiduously studied the cardiovascular system in many species (Ericsson et al. 2013). Thanks to his investigations, Harvey published several texts; among those, the most popular is “*De Motu Cordis*”, which describes in great detail the human circulatory system (Harvey 1737; Harvey and Keynes 1995).

France had a pivotal role in experimental biology and medicine during the 1800s. Notable scientists such as Claude Bernard (1813 – 1878 AD) aimed to establish the rules of the scientific method relying on animal experimentation (Fox 2015).

Even if Bernard's methods were considered brutal by some, including his wife and son, he is still considered a "true discoverer", since he was the first scientist referring to the "*Milieu Intérieur*", which would later be called homeostasis (Cannon 1929; Cooper 2008). In 1865, Bernard published his major work on the description of the scientific method, entitled "*An Introduction to the Study of Experimental Medicine*" (Bernard 1865).

The discovery of vaccination as a technique to counteract infectious diseases, dates back to 1796 by the British doctor Edward Jenner (1749-1823), who developed the smallpox vaccine using different animal species (**Figure 1**) (Gross and Sepkowitz 1998). Louis Pasteur (1822 – 1895 AD) observed that his studies on animal diseases could bring benefit on both animal and human health, enhancing the understanding of human diseases and pathologies (Ericsson et al. 2013).

From the beginning of the twentieth century, the use of animals for scientific purposes increased drastically and, even if with some criticism by the public opinion, animal modeling, especially with rodents, had become the preferred method for demonstrating scientific significance. The remarkable strength of the experimental approach led to what has been called the Golden Age of scientific medicine (Fox 2015). When the respectable surgeon Alexis Carrel was acknowledged with the Nobel Prize in 1912, the statement read: "you have proved once again that the development of an applied science of surgery follows the lessons learned from animal experimentation" (Hamilton 2016). In 1929, another Nobel Prize winner in Physiology and Medicine, August Krogh, introduced the concept of "the comparative method", stating that it should be chosen, when designing an animal experiment for biomedical purposes, the animal species in which the disease occurs naturally, mimicking the human illness (Krogh 1929).

It can be finally remarked that, developing from ancient times through the present day, animal experimentation and animal modeling have been fundamental pillars of the scientific community, leading to big part of the current biological and medical knowledge.

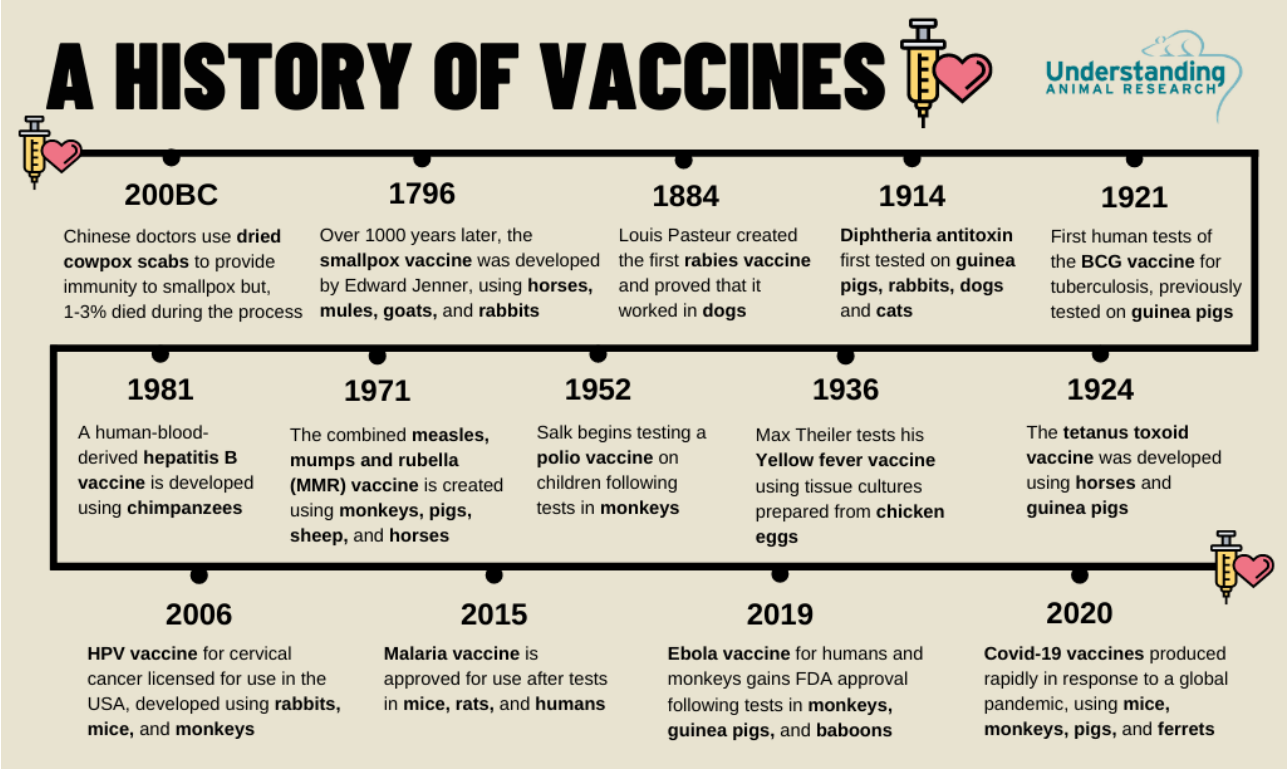

Figure 1. **History of vaccines timeline**. Adapted from Understanding Animal Research website: https://www.understandinganimalresearch.org.uk/resources/infographics.

1.2 Research culture, legislation and ethics

The term Laboratory Animal Care refers to the application of veterinary medicine to laboratory animals, aiming at improving their husbandry and management. The expression also refers to the treatment of pain, distress and disease of the species used for animal research, provided by a specialized veterinarian (Fox 2015). One could assume that the history of animal research regulation would have been simultaneous to the development of a meaningful social ethic; indeed, some philosophers from the past, such as Plutarch, Schopenhauer and Bentham, have mentioned humans' ethical obligations towards other animal species (Preece 2006). Nevertheless, the history of animal care and ethics is considered relatively scarce. The earliest laws against cruelty towards non-human beings were just to counteract sadist showing extremely brutal behaviors (Rollin 2006). Then, during mid-twentieth century, the western society started to demand for animal ethics regulations. In the US, this period coincided with a massive rise in experimental fundings from the National Institute of Health (NIH) and the set up of large amounts of toxicity testing. In 1966 the "*Animal Welfare Act*" was published, allegedly to protect (some) animal species used within biomedical research; unfortunately, the legislation specifically excluded mice, which was and still is the most used species in animal experimentation (Rollin 2019). In 1985 a new amendment laid the basis for the control of pain and distress in all animals enrolled for research purposes (Rollin 2006).

On the other side of the Atlantic Sea, in Great Britain, politician Richard Martin obtained the approval of a law to prevent cruelty and improper treatment of cattle already in 1822 (Turner 1980). The Brambell commission is still recognized, worldwide, as a moral lighthouse even if was developed for animals bred for agricultural purposes. Most famously, the Brambell report was responsible for the concept of the Five Freedoms, explained by the Farm Animal Welfare Council in 1965; the authors stated "The welfare of an animal includes its physical and mental state and we consider that good animal welfare implies both fitness and a sense of well-being", introducing the good mental state to fulfill animal well-being (Brambell and Systems 1965).

In the last decades, the most remarkable and international change regarding animal experimentation regulation has been the increasing interest to the development of a Culture of Care amid research establishments and facilities (Davies et al. 2018). A precursor of these more social and cultural questions regarding Laboratory Animal Care is represented by the Russell and Burch's book published in 1959; their work focused on psychological, sociological and organizational factors affecting the embracement of existing knowledge to improve experimentation (Russell and Burch 1959).

One aspect of western society ethics concerns the problem of weighing the individual interests against general welfare. The essential interests of the human beings are called rights and are protected by moral and legal fences. In 1986, within the Council of the European Communities, it was approved one of the earliest examples of common legislation establishing equal standards for the use of animals in research across the Member States of the former European Economic Community (European Directive 86/609/EEC) (Olsson et al. 2016). Almost two decades later, the European Union started a revision process of the animal experimentation legislation; this review resulted in the Directive 2010/63/EU (Commission 2010). The new legislation asks for explicit justification about the selection of the animal species, the methods, the

scientific rationale and the harm-benefit analysis; authorization of the experimental protocol by National Authority is mandatory (Commission 2010).

One of the 2010/63/EU Directive objectives is to ensure high standards of laboratory animal welfare across all Europe; non-technical summaries, retrospective reviews and statistical reports are part of the transparency measures to assist scientific communities strive for excellence and support open communication with the society (Chlebus et al. 2016)**.**

Lab Animals Veterinarian Organizations are pivotal as a means of communication between colleagues from all around the world, sharing experiences and providing suggestions for the common issues that arouse within this field of veterinary medicine. In 1949 the American Association for Laboratory Animal Science (AALAS) was founded, firstly named Animal Care Panel (**Figure 2**) (Fox 2015).

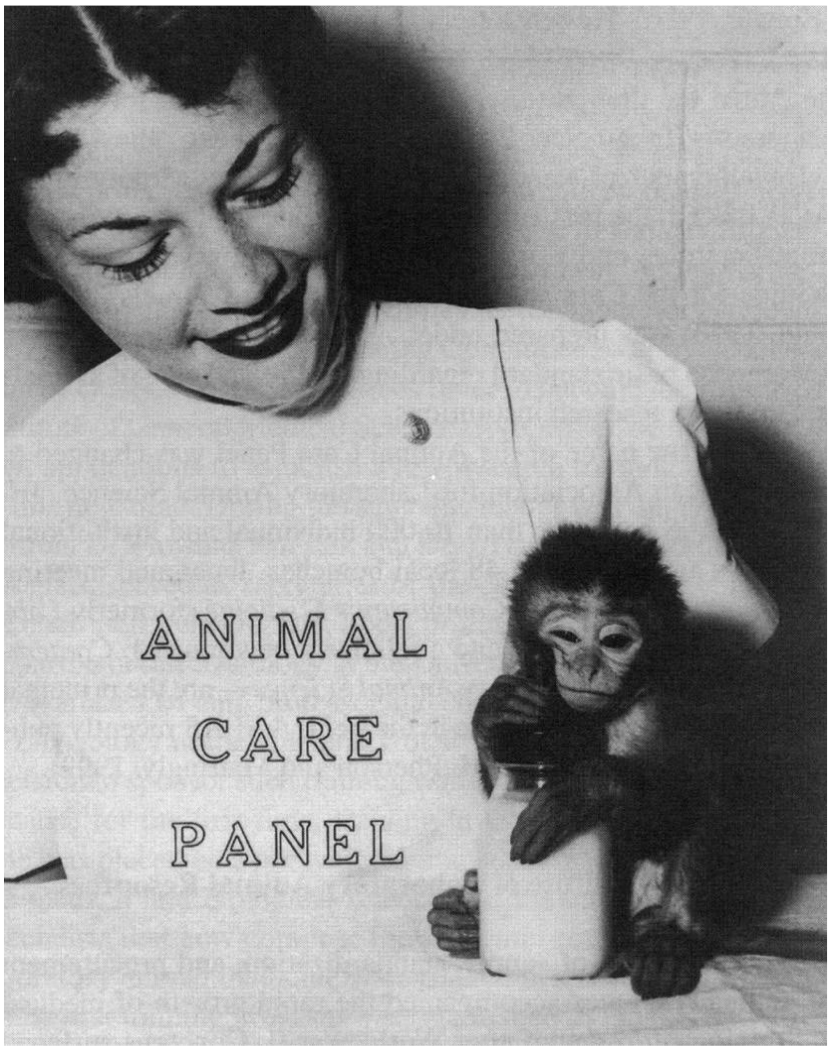

Figure 2. Cover of early descriptive brochure about the Animal Care Panel (1950s), now the American Association for Laboratory Animal Science. Adapted from "*Laboratory animal medicine*" page 14; Elsevier (2015).

As of today, many organizations can be found worldwide, with an array of acronyms: AAALAC International, ACLAM, ECLAM, ESLAV, and so on. The Federation of European Laboratory Animal Science Associations (FELASA), established in 1978, has a membership of 21 independent European national and regional laboratory animal science associations; members are usually animal scientists, technologists and veterinarians (https://felasa.eu/).

In conclusion, even if a lot of attention has been given to animal welfare especially since the mid twentieth century, the use of animals in biomedical research still represents a subject of heated debate. The first Society for the Prevention of Cruelty to Animals (SPCA) was established in England, followed in the 1860s by the American SPCA; unwillingness to the use of animals in science were part of the concerns of these societies (Fox 2015).

Extremist opponents, such as anti-vivisectionist groups, are usually against any kind of animal use, arguing that Animal Experimentation is vicious and not necessary, despite of its benefits. However, as briefly seen in the previous chapter, animal research has contributed to many of the medical advances we now take for granted. All humanity, as well as non-human animals, have benefited from vaccines, antibiotics, anesthetic drugs and innovative surgical techniques.

Lastly, animal research allowed humans to deal with newly emerged infectious diseases, such as HIV and COVID-19 (Veazey and Lackner 2017; Witt et al. 2021). Using animals within experiments is still fundamental to provide either significant biological knowledge or improvements in human and animal health; to comply with high quality scientific results, the highest standards of animal welfare should be maintained.

1.3 Replacement, Reduction, Refinement (3Rs)

The number of animals enrolled in experimental protocols for the first time within the Member States of the European Union between 2015 and 2017 is below 10 million subjects annually; specifically, the last EU Parliament and Council report listed 9.39 million of animals used for research, testing, routine production and educational (including training) purposes in 2017 (**Table 1**). The main species used were mice, fish and rats, accounting together for the 86% of the total number of animals, while pigs accounted for approximately 0.13% (**Figure 3**).

(*Report From The Commission To The European Parliament And The Council 2019 report on the statistics on the use of animals for scientific purposes in the Member States of the European Union in 2015-2017*, 2020).

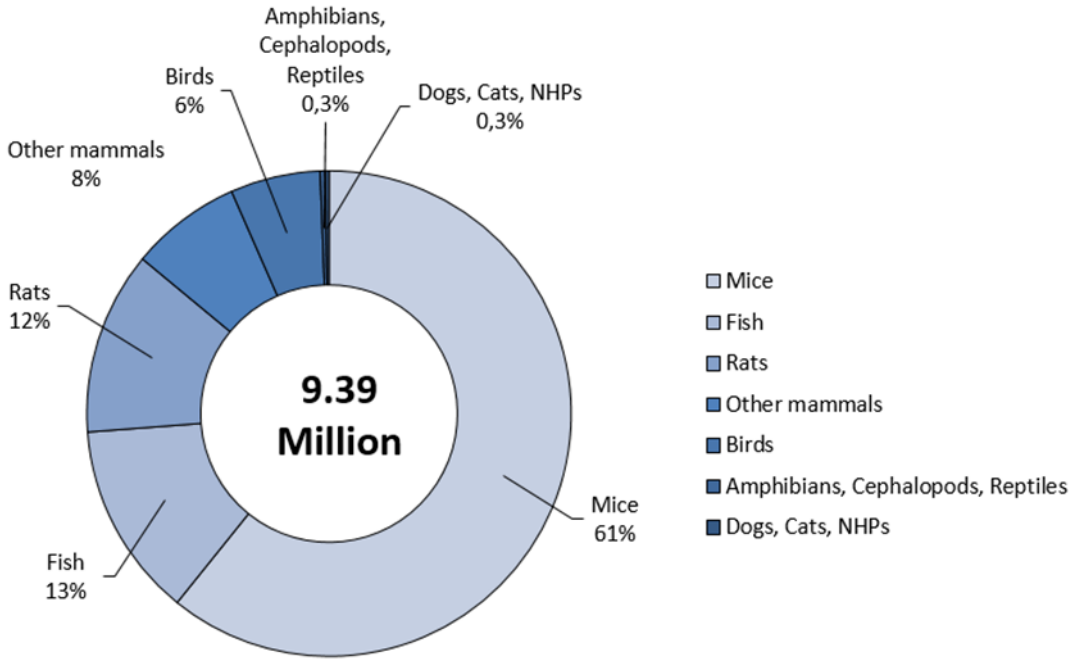

Figure 3. Numbers of animals used in biomedical research by main classes of species in 2017; Adapted from EU report 2015-2017, 2020.

	2015	2016	2017
Mice	5,711,612	5,989,413	5,707,471
Rats	1,201,189	1,173,135	1,146,299
Guinea-Pigs	149,328	150,985	144,824
Hamsters (Syrian)	20,195	18,614	12,700
Hamsters (Chinese)	30	519	187
Mongolian gerbil	6,199	5,645	5,239
Other rodents	26,088	13,712	25,172
Rabbits	346,052	350,405	351,961
Cats	1,975	1,951	1,879
Dogs	14,501	15,691	13,688
Ferrets	2,212	1,530	2,016
Other carnivores	3,648	1,444	2,386
Horses, donkeys and cross-breeds	3,217	3,474	2,414
Pigs	73,895	80,029	71,522
Goats	2,233	1,365	1,563
Sheep	20,106	21,240	18,812
Cattle	26,763	22,782	30,643
Prosimians	169	44	98
Marmoset and tamarins	429	285	465
Squirrel monkey	13	8	8
Other species of New World Monkeys (Ceboidea)	0	0	3
Cynomolgus monkey	6,221	6,503	7,227
Rhesus monkey	211	318	353
Vervets (Chlorocebus spp.)	56	19	33
Baboons	37	62	25
Other species of Old World Monkeys (Cercopithecoidea)	0	0	23
Other mammals	9,535	3,637	26,335
Domestic fowl	515,834	500,920	464,553
Other birds	119,377	94,804	99,410
Reptiles	2,414	3,240	2,937
Rana	4,884	4,482	3,485
Xenopus	10,837	18,511	13,539
Other amphibians	20,190	19,558	10,683
Zebra fish	338,815	513,011	499,763
Other Fish	936,252	791,726	719,932
Cephalopods	15,862	8,884	514
Total	**9,590,379**	**9,817,946**	**9,388,162**

Table 1. Numbers of animals used for the first time by species.
Adapted from EU report 2015-2017, 2020.

The second of the 3Rs "*Principles of Humane Experimental Technique*", published in 1959 by Russell and Burch, is Reduction; indeed, the authors highly suggested that every effort should be made to reduce to a minimum the number of animals used (Russell and Burch 1959). Since its release, this document has deeply influenced the European legislation aimed at regulating the use of animals for scientific purposes; the 3Rs principles have been established as essential considerations when animals are used in research. Within the 3Rs principles, the concept of Reduction could appear less argumentative, but its application has highlighted a considerable gap of appropriate statistical advice when drafting an experimental protocol. Common experts' opinion emphasizes the need to select the appropriate number of animals for each specific study upon a priori sample size calculation (Smith et al. 2018).

Russell and Burch's 3Rs provide a means to improve animal welfare; the others two principles are Replacement and Refinement. Regarding the former, the authors highlighted the need to Replace living animals with non-sentient alternatives, as much as possible (Russell and Burch 1959). However, in the last decades major problems have been encountered when replacing animals with *in vitro/in silico* methods; it has often proven difficult to formally validate the alternative, especially in regulatory toxicology and pharmacology (Flecknell 2002).

Nowadays, the most effectively implemented principle in experimental procedures and laboratory animal facilities is the concept of Refinement; as Russell and Burch stated in their publication, every effort should be made to Refine animal experiments so that they would cause the minimum pain and distress (Russell and Burch 1959). The idea of Refinement should be applied to the entire lifespan of a laboratory animal: how it is bred, transported, housed, cared for and handled, its general health and, lastly, the method of euthanasia employed. Notable changes have been made in these areas, such as the freedom from diseases, since infectious agents have been virtually eliminated from many research facilities; indeed, animals' health status is frequently monitored and they live in a carefully controlled environment, with standardized bedding and diets (Flecknell 2002; De Angelis et al. 2019).

Significant progress is being made with the introduction of reliable pain and distress evaluation methods and of more Humane End Points (HEPs) during experiments (Morton 2000). Pain can be assessed through objective and subjective scales based on animal pain-related behaviors analysis; this represents a non-invasive technique and does not require special equipment nor restraint, yet personnel has to be highly trained to avoid biases (Sotocina et al. 2011; Miller and Leach 2015; Luna et al. 2020). The definition of simple scores helpful for decision-making increase the confidence of specialist veterinarians and, maybe most importantly, of researchers who are usually unexperienced about animal pain assessment and treatment. The use of analgesics to control pain is becoming more widespread, enhancing laboratory animals welfare (Dobromylskyj et al. 2000).

It is current opinion that the adoption of the 3Rs principles can effectively improve the quality of science; appropriately designed experimental protocols and standardized optimal conditions of animal care that minimize unnecessary stress or pain, often produce better and more reliable scientific data (Burden et al. 2015). Both standardization and proper communication resulted in a worldwide reduced variability in research, leading to smaller group sizes of animals (Flecknell 2002; Burden et al. 2015; De Angelis et al. 2019).

In conclusion, it is self-explanatory how appropriate standardized planning will increase the likelihood of the research success and represents an important step in the implementation of the Russell and Burch’s 3Rs: Replacement, Reduction, Refinement.

2. The Swine Model

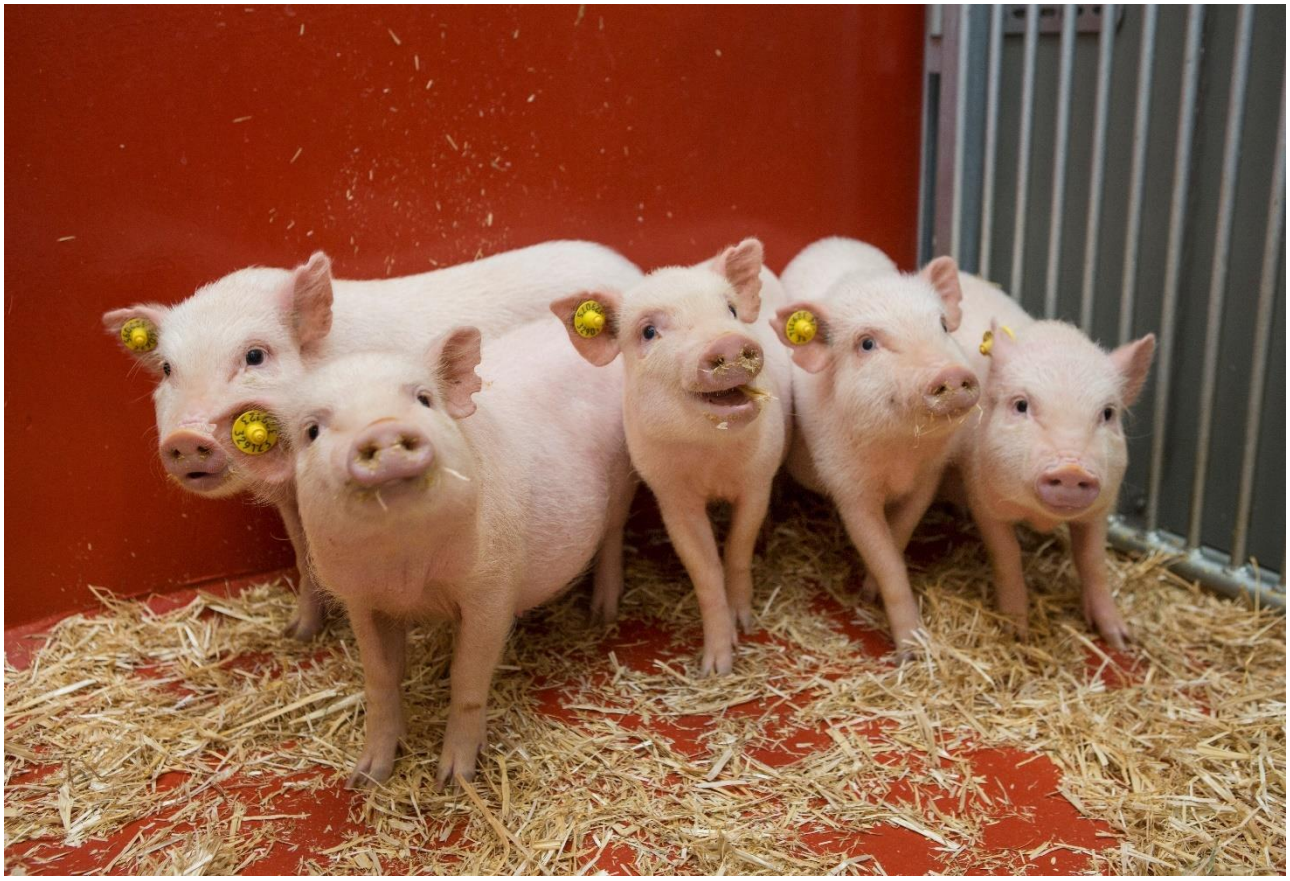

Figure 4. Göttingen Minipigs. Adapted from https://minipigs.dk/pictures.

2.1 Taxonomic Classification and Breeds

The porcine taxonomic classification is the following (Erxleben 1777):

- Phylum: Chordata
    - Subphylum: Vertebrata
- Class: Mammalia
- Order: Artiodactyla
    - Suborder: Suiforme
- Family: Suidae
- Genus: *Sus*
- Species: *scrofa*
- Subspecies: *domestica*

The pig, often referred to as swine or domestic pig when distinguishing from wild members of the genus *Sus*, is an omnivorous even-toed ungulate mammal (Michael Swindle and Smith 2008). Swine breeds can be classified into two main categories: standard farm breeds raised within zootechnical industry, and miniature breeds; the most important difference concerns the size of the animals at adult age (Helke et al. 2015). Common standard large breeds commercially available within Europe are Large White, Landrace, Duroc, and crossbred hybrids (Bollen et al. 2010). These animals, raised mainly for meat production, are usually referred to as "domestic farm breeds" when used for biomedical research purposes, unless the specific breed used has a relevant importance for the model and needs to be clearly mentioned.

When drafting an experimental protocol with domestic farm breeds, major considerations to age and growth rate should be given. In this kind of research programs pigs are usually enrolled at a very young age, 8-12 weeks old, weighting between 15 to 30 Kg; indeed, domestic breeds weight at sexual maturity is approximately 80 Kg (5-6 months of age) (Swindle 2007). This is the reason why domestic swine are scarcely used for long-term projects or when adult to old animals are required.

Looking at availability, commercial farm breeds are easy to find almost worldwide; however, the health status of the different suppliers can vary extensively. For biomedical research pigs should be designated SPF, Specific Pathogens Free (Swindle 1996). Nonetheless, the accreditation SPF does not mean that the animals are free from other infectious agents that might interfere with the research results; indeed, it would be considered good practice for scientists to discuss with the institutional veterinarian for potential research-jeopardizing diseases (Swindle et al. 1994; Swindle 2007).

As previously stated, the main difference between commercial farm breeds and miniature pigs is size at sexual maturity. The latter are usually either naturally developed or raised on purpose for research or pet keeping; indeed, miniature pigs provide an overall smaller porcine model that could be easily manageable at sexual maturity when compared to the conventional bigger cousin (McAnulty et al. 2011).

Miniature breeds are usually specified within scientific papers and can be distinguished from micro-pigs, which are even smaller in size (Michael Swindle and Smith 2008). The more commercially available breeds mentioned in recent biomedical literature are: the Yucatan (**Figure 5**), the Göttingen (**Figure 6**) and the Hanford (Swindle 2007).

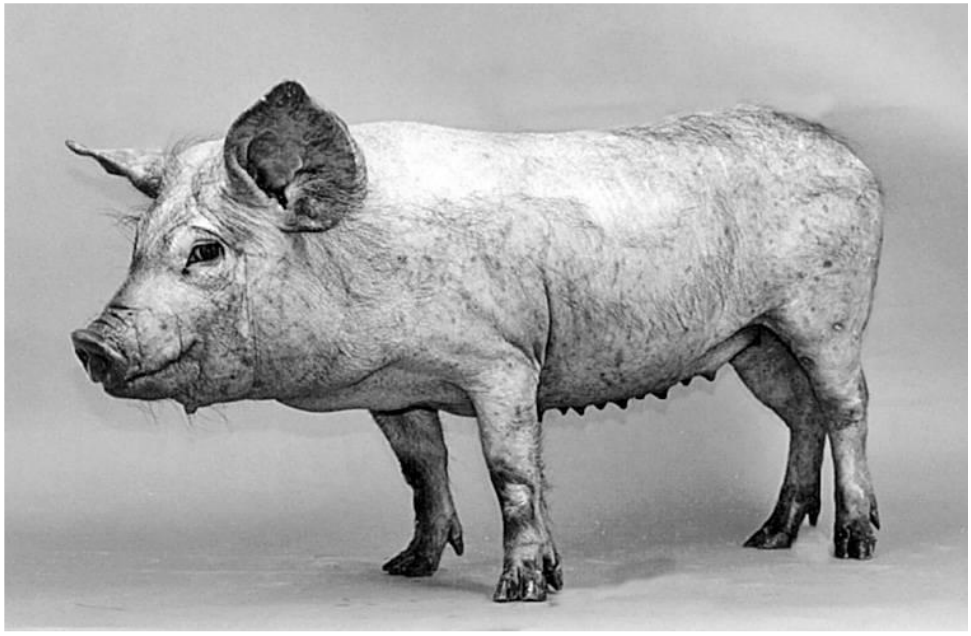

Figure 5. Yucatan™ miniature pig. Adapted from Swindle, M. M. (2007). *Swine in the laboratory: surgery, anesthesia, imaging, and experimental techniques*. CRC press.

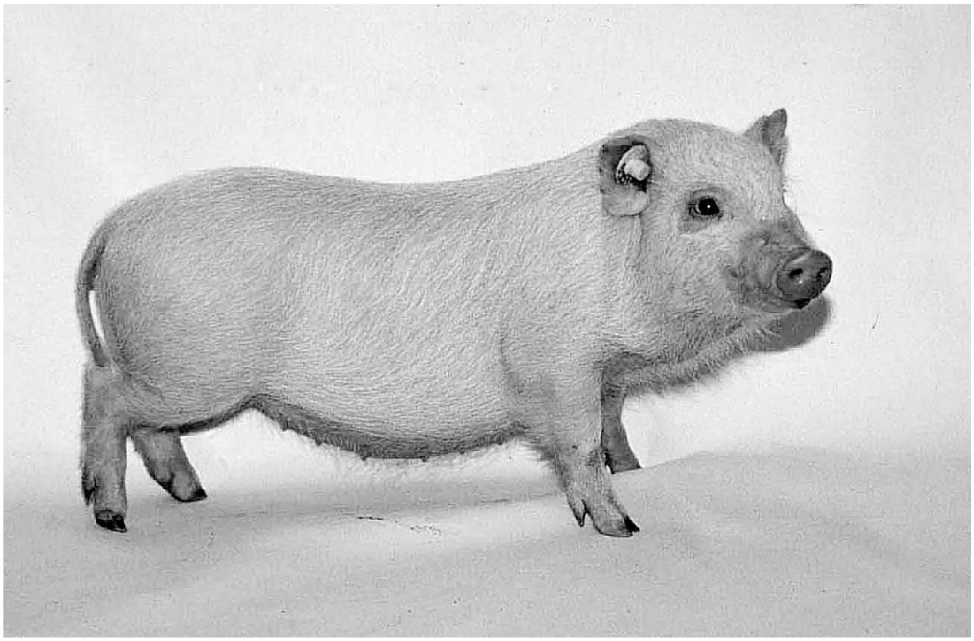

Figure 6. Göttingen Minipig®. Adapted from Swindle, M. M. (2007*).* *Swine in the laboratory: surgery, anesthesia, imaging, and experimental techniques*. CRC press.

Adult minipigs range from 30 to 50 kg in body weight (**Figure 7**) and, consequently, are more manageable than larger commercial breeds for long-term projects. Furthermore, miniature pigs purchased from commercial breeders of laboratory animals usually come with higher health status than that of SPF farm animals (Swindle et al. 2012; Helke et al. 2015).

In the last decade, the swine species has become a strong model for surgery, both in teaching and research, and for translational studies of specific disease conditions, due to the anatomic and physiological similarities to humans (Helke et al. 2015).

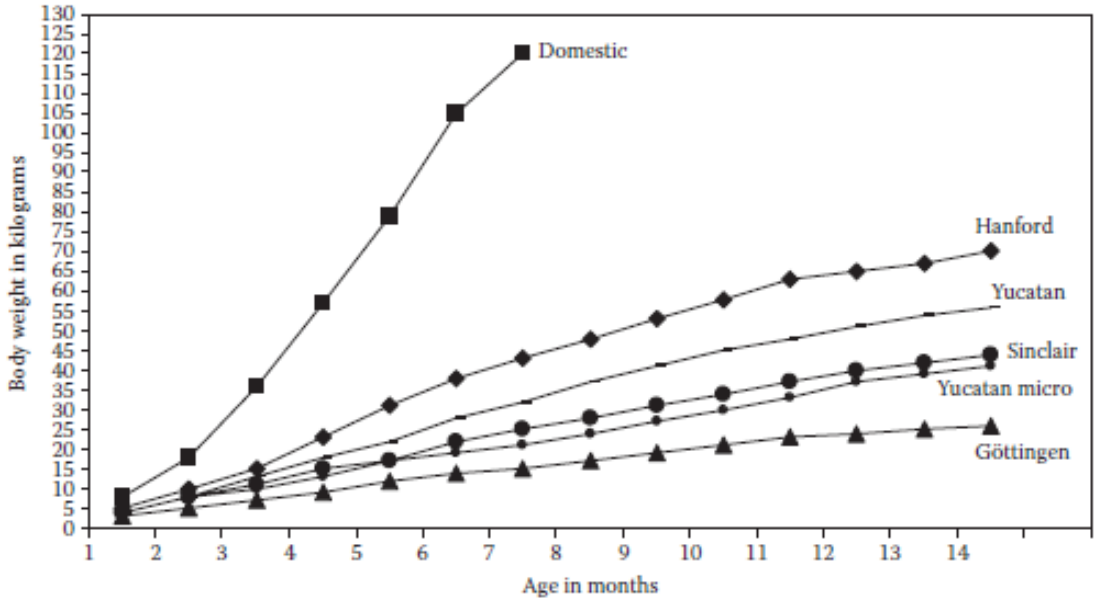

Figure 7. Relative Growth Rates of multiple Swine breeds.

Adapted from Swindle, M. M. (2007). *Swine in the laboratory: surgery, anesthesia, imaging, and experimental techniques*. CRC press.

2.2 General Biology

Generally speaking, swine undergo puberty between 3 to 7 months of age, with miniature breed subjects becoming sexually mature already at 4-6 months of life (Swindle 2007). Females are polyestrous and their estrous cycle lasts around 21 days, ranging from 17 to 25 days (Helke et al. 2015). During the estrus, which lasts about 48 hours, sows show a typical "freezing" behavior when moderate pressure is applied to their rump (Helke et al. 2015).

The gestation period of both miniature and commercial farm breeds lasts 114 ± 2 days, while litter size strongly depends on the breed, with domestic large swine usually having 8 to 12 piglets on average and miniature sows 4 to 6 (Swindle 2007; Flowers 2020). Once started, farrowing typically ends in 4 hours, ranging from less than 1 up to 8 (Flowers 2020). The swine species is characterized by a diffuse epitheliochorial placenta, which is the reason why newborns must consume colostrum within the first day of life to get maternal immune protection before their intestine loses the capability to absorb immunoglobulins (Fox 2015).

Piglets do not have brown fat at birth and their physiological ability to thermoregulate develops over the first week of life; this is the reason why newborn pigs require an heat source to prevent cold stress (Flowers 2020). The temperature in the nest should reach 29-35°C (85-95°F) through the use of a supplemental heat lamp (**Figure 8**), which must be placed just for the neonates because on the other hand, the sows' comfort temperature is around 21°C (70°F) (Council 2010).

Piglets are mobile shortly after birth and in the first week sows nurse them almost hourly (Helke et al. 2015). For what concerns the duration of lactation, the European Council Directive 2008/120/EC states that: "*no piglets shall be weaned from the sow at less than 28 days of age unless the welfare or health of the dam or the piglet would otherwise be adversely affected"* (2019).

Figure 8. The picture shows a delivery pen where the piglets have easily access to the sow and are provided with an infrared heating lamp.
Adapted from Professional Pig Community: https://www.pig333.com.

The zootechnical industry requires extremely high reproductive performances from the sows, yet, due to poor maternal iron storages, piglets are prone to develop severe iron deficiency that could lead them to anemia (Venn et al. 1947). This condition occurs regardless of the breed and it is therefore mandatory to administer exogenous iron-dextran to newborns, preferably within the first days of life, to prevent anemia (Starzyński et al. 2013).

Long bones epiphyses are not completely sealed until 3.5 years in domestic swine breeds; while for miniature breeds, epiphyses closing time varies depending upon the size of the animal (Swindle and Smith, 2008). Even though the life span of domestic swine in the intensive farm system is below 6 months for meat production and below 5 years for breeding stock animals, their "natural" life span might range between 15 to 25 years, depending on the breed and environmental conditions (Swindle 2007).

Pigs are considered true omnivores and will consume a wide variety of foods in the wild (Pond and Lei 2001). The porcine gastrointestinal tract has peculiar anatomical features. The majority of the large intestine is arranged forming a spiral in the left upper quadrant of the abdomen; these centripetal and centrifugal coils are composed of cecum, ascending, transverse and part of the descending colon (Swindle 2007). In spite of the anatomical differences, the swine gastrointestinal physiology is very similar to humans'; the same is for what concerns the cardiovascular system, especially regarding the coronary arteries anatomy (Helke et al. 2015).

Domestic farm breeds swine, raised for meat production, require metabolizable energy (ME) dependent on the different weights: 3265 kcal/day (10-20 Kg), 6050 kcal/day (20-50 Kg) and 8410 kcal/day (50-80 Kg) (Pond and Lei 2001). Diets for miniature breeds should have higher content of fiber and less amount of fat and proteins, since these animals tend to become obese easily, especially females (Bollen et al. 2010). When formulating a pig diet, Vitamin E and selenium content need to be checked as their deficiency could lead to cardiac and hepatic pathology (Pond and Lei 2001). Feeding should be given once or twice a day and never *ad libitum*, while fresh and clean water must be always available; pigs require water amount of 2.5 L for each Kg of feed consumed (Pond and Lei 2001).

Swine hematologic parameters vary to some degree depending on environmental conditions, health status, breed, age and gender; nonetheless, physiological values are generally comparable, and tables of ranges from various pig breeds are available in notable textbooks (Swindle 2007; Helke et al. 2015). Compared to other mammalian species, the swine hematocrit tends to be physiologically lower; furthermore, they usually have equal percentages of lymphocytes and neutrophils (Ventrella et al. 2017). The blood volume of adult pigs is around 65 mL/Kg; their blood clotting pathway could be considered similar to humans' but swine blood group antigens are 16, A-P (erythrocyte antigen A to P (EAA-EAP)) (Swindle 2007).

Differences between human and swine physiological parameters were found, such as plasma volume, arterial pH, core temperature and others, part of which have been attributed to the immature age of the pigs studied (Hannon et al., 1989).

Pigs show complex behavioral patterns, they are highly social and intelligent animals. Swine rely mostly on their notable senses of smell and hearing, in respect to the poor eyesight (Swindle et al. 1994). Due to their innate curiosity, these animals tend to show rooting behavior and they can become destructive if adequate enrichment/stimuli are not provided (Helke et al. 2015). Swine can be easily trained with positive reinforcement, usually provided by food rewards (Thomas 1991; Paredes-Ramos et al. 2020). Training pigs in research settings is extremely useful to accustom animals to be handled and restrained without stressing them.

2.3 Current use in Translational Medicine

The relationship between pigs and humans prospered throughout history because of the swine high adaptability to different environments and needs of humans (Pond and Lei 2001). One major goal of animal experimentation is to transpose data from animal trials to benefit human clinical research; indeed, before initiating human clinical studies, animal models are investigated to evaluate the safety, and subsequently, the efficacy of new drugs, procedures and devices (Smith et al. 2018). Nowadays, swine models have replaced the majority of the earlier canine models (Robinson et al. 2019).

In biomedical research, the porcine species have been used mainly to study the cardiovascular system, because of their similarity to humans in anatomy and physiology (Crisostomo et al. 2016). Cardiovascular diseases in which the pig represents a useful model include atherosclerosis, congenital heart disease, heart failure, and testing of interventional devices, such as grafts and stents (Swindle 2007; Crisostomo et al. 2016).

Organ transplantation research has been proven particularly suitable with pigs, providing a model where different organs can be studied within the complexity of the *in vivo* environment. Swine transplantation trials have been performed on the heart, lung, liver, kidney and viscera (Swindle et al. 1994; Swindle 2007). Indeed, these animals are being studied as donors for xenotransplantation (**Figure 9**) (Swindle et al. 1994; Reardon 2022), which has determined the development of transgenic strains (Webster et al. 2005; Manzini et al. 2006).

Nephropathies represent another field of research in which swine have been enrolled; they have been used as models of renal hypertension, ureteral and intrarenal reflux, and urinary obstruction (Helke et al. 2015).

Figure 9. Pig model for heart xenotransplantation. Adapted from Understanding Animal Research website: https://www.understandinganimalresearch.org.uk/resources

The organs size, the anatomy and the response to immunosuppressive therapy make pigs ideal for many surgical models. Indeed, they are increasingly used in research and teaching protocols that concern surgery (**Figure 10**) (Swindle 2007). Swine are chosen as models for most of the laparoscopic and endoscopic experiments, innovative catheter insertion procedures to deliver interventional devices or drugs have been extensively studied as well (Lambertini et al. 2015; Crisostomo et al. 2016).

Figure 10. Göttingen Minipig® with back dressing after surgery. Adapted from Understanding Animal Research website: https://www.understandinganimalresearch.org.uk/resources

When it comes to neuroscience, the swine brain resembles the humans' according to weight, volume, cortical surface area, myelination and electrical activity, and its maturation extends from prenatal to first postnatal life just like the human brain (Lind et al. 2007). Techniques for swine spinal catheterization have been recently developed and are highly useful in different research areas, including anesthesia, analgesia and gene therapy delivery (Lambertini et al. 2015; Ventrella et al. 2016).

Swine models of retinal diseases have been frequently used in translational studies, thanks to pigs ophthalmological similarities to humans. As an example, the iodoacetic acid model of photoreceptor degeneration is used to mimic to a great extend the phenotype of human retinal pathologies (Barone et al. 2018b; Elmi et al. 2019; Barone et al. 2020; Ventrella et al. 2022).

For food related products and additives safety trials, the porcine model has been largely used. Swine are enrolled in nutritional and gastrointestinal researches since their physiology of digestion and their omnivorous diet resembles the humans' (Pond and Lei 2001). Domains of translational studies with pigs comprehend nutrient absorption, hepatic metabolism, parenteral nutrition and gastroenteric diseases such as necrotizing enterocolitis and gastric ulceration (Bryszewska et al. 2017; Barone et al. 2018a).

Swine and humans share similar membrane transport mechanisms and enzymes; indeed, porcine models for toxicology testing and drug disposition have already been developed (Helke and Swindle 2013). Furthermore, when comparing to rodents, pigs and minipigs allow for larger volumes of multiple body fluids collection and tissue biopsies, helping to the development of translational studies (Swindle et al. 2012).

In conclusion, to provide meaningful results the use of the most relevant animal model should be mandatory; extensive literature knowledge is required for the selection of the more appropriate animal species, which would ensure study integrity and meet its scientific aim. Moreover, using the most suitable animal species will enable the employment of fewer living animals, fulfilling the 3Rs principle of Reduction (Burden et al. 2015).

Aims of the Study – The ConcePTION Project

Key objectives of the project

The major goal of the ConcePTION project is to organize a reliable platform that could systematically, efficiently and in an ethical manner, produce and distribute evidence-based informations to women and healthcare personnel regarding the effects of pharmacological compounds taken during breastfeeding. Through a large network this objective will be fulfilled by generating, recording, classifying and analyzing data from pharmacovigilance, preclinical modelling (*in vivo*, *in vitro* and *in silico*) and routine healthcare (**Figure 11**).

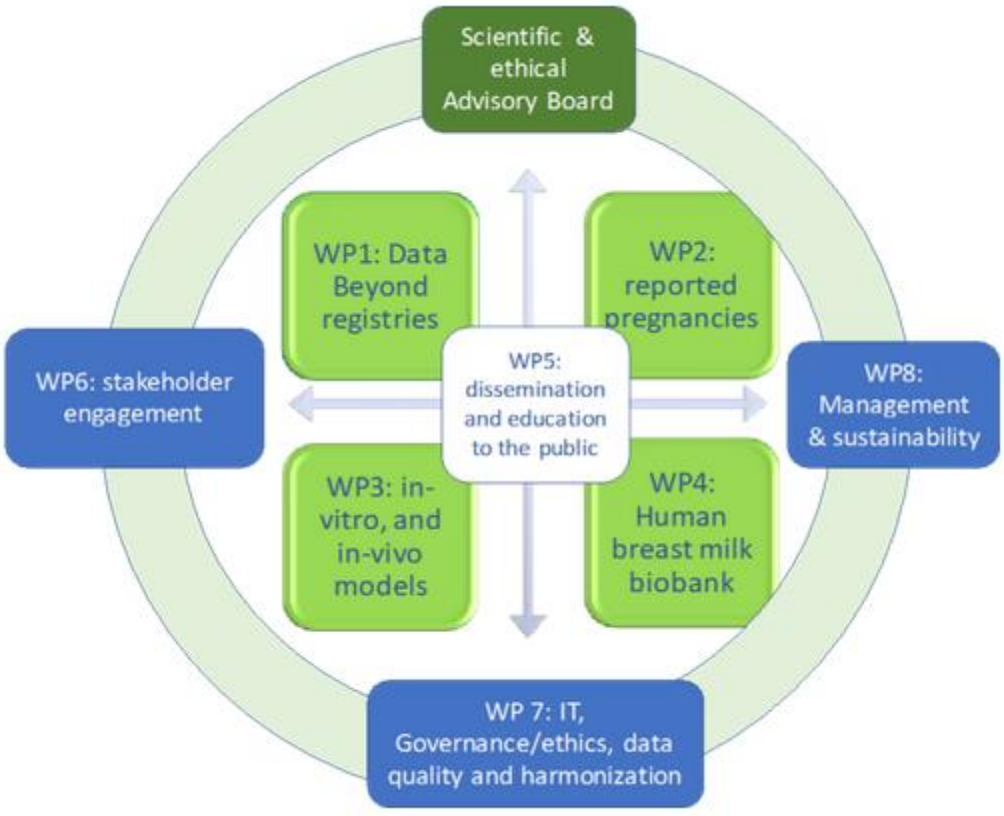

Figure 11. Project Design: ConcePTION is designed to have 8 work packages (WPs). Adapted from: https://www.imi-conception.eu

The ConcePTION project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement No 821520. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA (https://research-and-innovation.ec.europa.eu/funding/funding-opportunities/funding-programmes-and-open-calls/horizon-2020_en) .

ConcePTION unites 88 organizations from 22 countries; the Department of Veterinary Medical Sciences of the *Alma Mater Studiorum* – University of Bologna is within beneficiaries of the project and has active part in the Work Package 3 (WP3) "Determination of drug transfer and infant drug exposure during lactation: generation of quantitative and translatable data" (**Figure 12**).

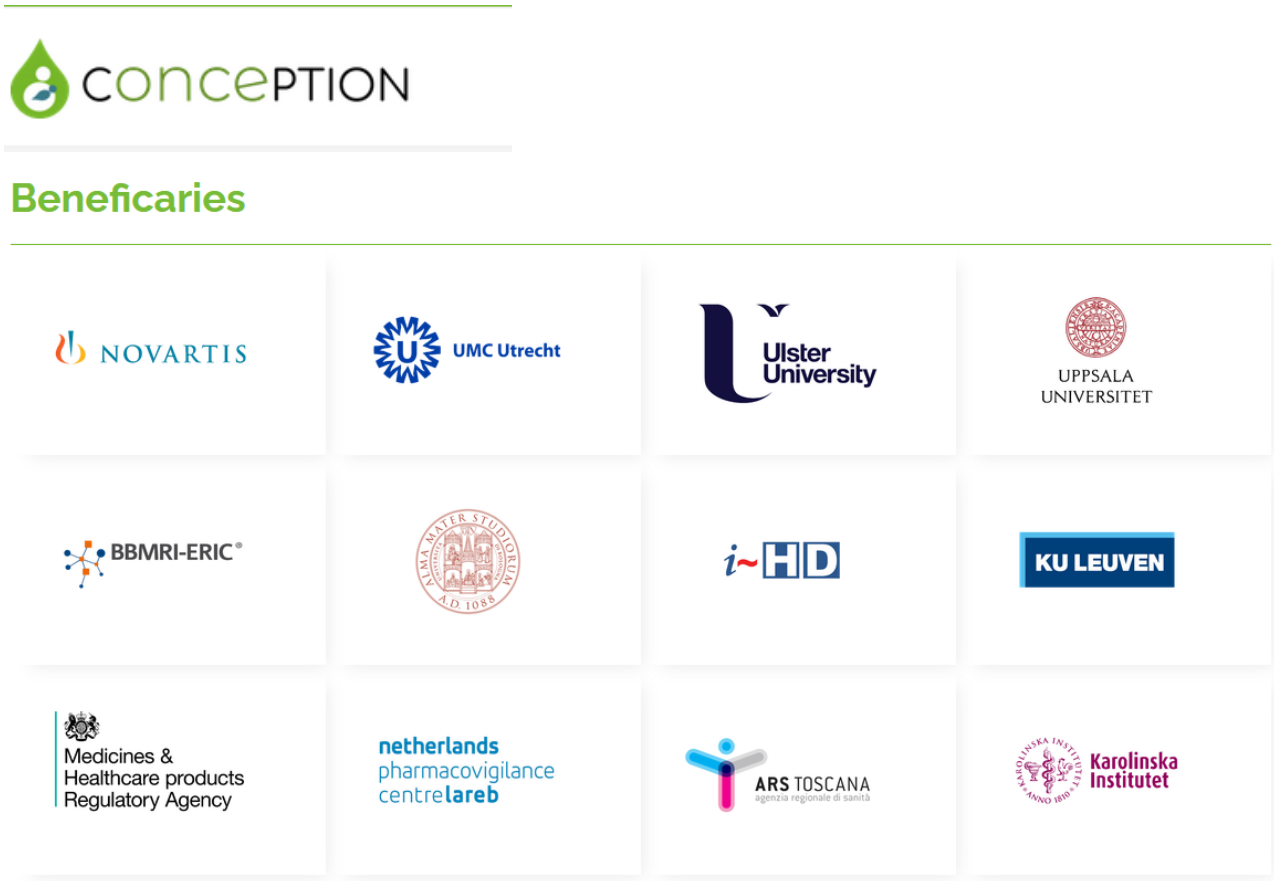

Figure 12. Some of the ConcePTION project Beneficiaries: Alma Mater Studiorum – University of Bologna included. Adapted from: https://www.imi-conception.eu

Main objective WP3

The objective of the Work Package 3 is to develop, characterize, validate and apply a non-clinical testing platform for evidence-based prediction of medication transfer in human breast milk together with drug exposure in breastfed infants.

Task 3.3 aims to build a reliable *in vivo* animal lactation model, along with an *in vitro* model, in a species sufficiently related to human reproductive and lactation physiology, and finally validate the translation of the animal model data to human clinical predictions. The *in vivo* model will give the opportunity to research on the impact of drug-specific properties, such as drug-transporters affinities, in determining the extent and rate of drug transfer in human breast milk, along with rapid determination of plasma-milk transfer rates of medications (metabolites included).

Relying on non-clinical data produced in WP3, it would be possible to build a Physiology-Based PharmacoKinetich (PBPK) modelling platform, to predict drug excretion rates in human breast milk and to determine systemic drug exposure in infants; PBPK-based prediction will comply with physiological factors such as milk composition, mammary epithelial cells transporters and gastrointestinal maturation of the newborn.

Informations generated within this WP3 will be beneficial to other WPs, regarding the use of these new predictive tools (*in vivo*, *in vitro* and *in silico* models) as valuable practices to inform risk assessment on drug use during breastfeeding.

Want to know more about the ConcePTION approach to study medicines in breastfeeding? Watch this clip: https://youtu.be/vubSYa0zVBQ .

This doctoral project develops within Task 3.3 of the WP3 of ConcePTION, aiming to develop an innovative animal model, translatable to humans, to perform *in vivo* lactation trials prior to drug approval.
The project is structured as follows:

1. **Preliminary step:** focused on performing an extensive bibliography review to obtain a better and wider understanding of the lactation physiological characteristics in the most used animal species for biomedical research. The outcome of the study led to the selection of the porcine species as a suitable candidate to set up the *in vivo* studies.
   - Deliverable 3.2 "*Report on lactation characteristics of animal species; Selection of the animal species to be used in in vivo studies*"
   - First Paper: Ventrella, D., Ashkenazi, N., Elmi, A., Allegaert, K., Aniballi, C., DeLise, A., ... & Bacci, M. L. (2021). "*Animal models for in vivo lactation studies: anatomy, physiology and milk compositions in the most used non-clinical species: a contribution from the ConcePTION project*." Animals, 11(3), 714.

2. **First step:** first *in vivo* trial performed at the *Alma Mater Studiorum* – University of Bologna on conventional farm hybrids and Göttingen Minipigs. Amoxicillin was chosen as first compound since it is a safe and widely used drug in porcine medicine. The study was conducted successfully and led the basis to the development of an innovative and reproducible swine model for pharmacokinetics translational studies during lactation.
   - Deliverable 3.3 "*Report on characterization in vitro human or animal mammary epithelial cell cultures models, including comparison between in vitro models*"
   - Deliverable 3.5 "*In vivo data on lactation transfer in one or more animal species*"

3. **Second step:**
    - Second Paper: Nissen, L., Aniballi, C., Casciano, F., Elmi, A., Ventrella, D., Zannoni, A., ... & Bacci, M. L. (2022). "*Maternal amoxicillin affects piglets colon microbiota: microbial ecology and metabolomics in a gut model*". Applied Microbiology and Biotechnology, 1-20.

1. Preliminary step

The objective of this preliminary step was to undergo through an accurate literature scanning and discern, by lactation characteristics, the most suitable animal species to perform in vivo studies to evaluate drug transfer into milk. Species providing higher translatable value to humans were considered: rodents (rats and mice), rabbits, dogs, (mini)pigs and non-human primates (NHPs).

Key topics for the literature search were the anatomy of the mammary tissue and the physiology of lactation, inclusive of qualitative and quantitative composition of milk.

As suspected the discrepancies between the different species, from an anatomical point of view, were considerable, but mainly regarding the gross number of mammary glands and teats. While on the other hand, looking at the physiology of lactation, no major variations were underlined and with prolactin and oxytocin as pivotal hormones for milk production and secretion.

The study of the literature regarding milk compositions between the different mammalian species was, to a certain degree, hard to interpret. Indeed, several research articles were old and relied on outdated analytical tools. Again, it was the smaller species that showed higher differences in milk composition compared to humans.

To conduct the present literature analysis, additional practical aspects were considered, such as ethics, social and financial implications and, in conclusion, the overall feasibility of lactation trials. All the authors agreed on the complexity of the development of an animal model for in vivo lactation studies. The swine species was selected as the most suitable choice when considering ethical factors, milk volume, litter size, ease of sampling, and translatability value to the human species.

- Deliverable 3.2 "*Report on lactation characteristics of animal species; Selection of the animal species to be used in in vivo studies*"

IMI2 821520 - ConcePTION

ConcePTION

WP 3-Determination of drug transfer and infant drug exposure during lactation: generation of quantitative and translatable data

D3.2 Report on lactation characteristics of animal species; Selection of the animal species to be used in *in vivo* studies

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IMI2 821520 - ConcePTION

ConcePTION

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Document History

Version	Date	Description
V1.0	20 Jan 2020	First Draft
V2.0	03 Feb 2020	Second Draft for WP3 review
V3.0	11 Feb 2020	Third Draft for Management Board review

Abbreviations

FIL	Feedback inhibitor of lactation
GH	Growth hormone
LA	Lobuloalveolar
MECs	Mammary epithelial cells
NHPs	Non-human primates
PRL	Prolactin
TDLU	Terminal duct lobular unit
WP	Work package

Content

Abstract

The aim of this Task (3.1) was to perform an accurate literature scanning in order to gain knowledge regarding the lactation characteristics of the most used animal species in biomedical research settings. Resulting data used to select a potential candidate species for *in vivo* studies to evaluate drug transfer into milk during lactation with a high translatable value to the human setting. Species taken into account were the most commonly used in regulatory toxicity and included rodents (rat and mice), rabbits, dogs, (mini)pigs and non-human primates (NHPs). Reference data regarding humans were also collected and analyzed in order to highlight critical similarities and differences with the studied species. Ruminants were excluded from the search since their peculiar gastrointestinal anatomy and physiology results in a relatively lower translational value when (drug) metabolism is involved. Key topics were anatomy of the mammary gland, physiology of lactation and qualitative and quantitative composition of colostrum and mature milk. As expected, from a gross anatomy point of view, the variations between the analyzed species in terms of number of glands were very high, with only NHPs showing the same number as humans, despite differences in the number of canals. Nonetheless, having a higher number of mammary glands and teats, would allow for easier sampling procedures and higher volume specimens, without disturbing the animal in the feeding process. Regarding the physiology of lactation, no major discrepancies were identified amongst the analysed species aside from some differences in the production and overall physiology of prolactin in rodents. Aside from that, hormonal inputs and pathways were mostly conserved, with prolactin and oxytocin being pivotal. The comparison of colostrum/milk compositions between the different species was relatively hard to interpret in light of several technical and physiological issues. Indeed, a lot of the retrieved papers were old and relied on poor sample sizes and different, often outdated, analytical tools. Moreover, high inter individual variations within the same species were extremely common. Overall, smaller species showed higher differences in milk composition in comparison to humans. Additional practical considerations were also taken into account, such as ethical consideration regarding the chosen species which affects the group size, financial implications and technical feasibility of lactation trials (e.g., ease of sampling, volume of sampling, husbandry requirements and scientific recognition). In conclusion, the present analysis of the literature confirmed the complexity of the decisional process behind the choice of an animal model for *in vivo* trials. For some of the evaluated species, data was either poor or missing, highlighting the necessity to generate more physiological background studies for species that are routinely used in laboratory settings. Overall, when taking into consideration ethical factors, feasible group size, milk volume and ease of milk collection, and physiological similarities with humans, the Göttingen Minipig seems to represent the most appropriate choice.

Methods

A specific aim of the IMI ConcePTION work package (WP)3 was the collection of quantitative data in an animal model regarding the potential passage of pharmacological compounds and their metabolites into milk. Thus, literature searches were performed to identify a candidate non-clinical species relevant to human for use in lactation study(ies) and experimental trials. Species taken into account were the most commonly used in regulatory toxicology and included rodents (rat and mice), rabbits, dogs, (mini)pigs and NHPs. Reference data regarding humans were also collected and analysed in order to highlight critical similarities and differences with the studied species. Ruminants were excluded from the search since their peculiar gastrointestinal anatomy and physiology results in a relatively lower translational value when (drug) metabolism is involved. This would indeed create strong biases when considering the overall aim of the WP. The topic of the hereby presented report was divided into three main critical subcategories for the assessment of lactation characteristics:

- anatomy of the mammary gland
- physiology of lactation
- colostrum/milk qualitative and quantitative composition

A preliminary scanning of the literature highlighted a relative lack of relevant data in the most used databases such as PubMED and EMBASE, making a systematic approach unfeasible. Indeed, basic data regarding anatomy and physiology are often found in textbooks or old papers and used only as reference/control data in more recent studies. Therefore, it was decided to broaden the literature search to currently used Veterinary Medicine textbooks and to use less-specific search words and their combination for the different subcategories. Further literature searches were then performed starting from the reference sections of the retrieved articles. The search criteria are provided below:

- Anatomy of the mammary gland: preliminary search words included (“mammary gland” OR “udder”) AND (“anatomy” OR “morphology” OR “structure”) AND the different species afore mentioned and their synonyms.
- Physiology of lactation: preliminary search words included (“lactation” OR “milk production” OR “colostrum production”) AND (“physiology”) AND the different species afore mentioned and their synonyms.
- Colostrum/milk composition: preliminary search words included (“colostrum” OR “milk”) AND (“composition” OR “components” OR “quantitative composition”) AND the different species afore mentioned and their synonyms.

Retrieved articles, book chapters and books were then analysed to identify potential biases to the reported results as methodological errors or experimental design errors.

Results

Anatomy of the mammary gland

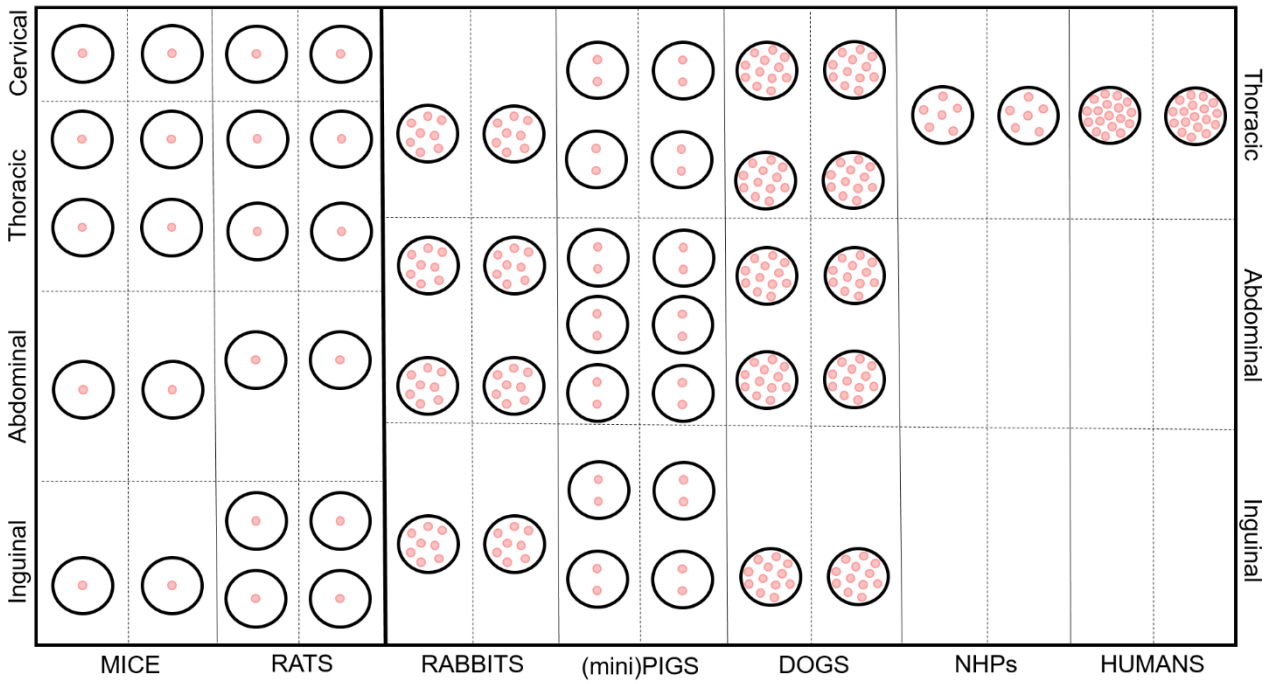
The results of the literature search regarding the anatomy of the mammary gland in the species taken into account for the present report are summarized in **Table 1**.

In general, mammary glands can be defined as modified glands that give name to the Mammal class, whose secretion is vital for the offspring survival. They are complex tubule-alveolar glands made of secretory units organized into lobules, surrounded by connective tissue septa [1]. From a developmental point of view, they originate as epithelial buds growing into the mesenchyme starting from linear ectodermal thickenings, also known as mammary ridges, and subsequently placodes [2]. Mesenchyme starts proliferating around such buds to create a teat/nipple on the skin surface. At this stage, epidermal sprouts start developing, from the buds to the teat/nipple, creating canals. Every canal will then create a separate duct that associates with a glandular mass and a separate orifice [1]. The number of overall glands, teats/nipples and canals vary amongst mammals as shown in **Table 1**, as well as the anatomical location of the mammary unit, represented in **Figure 1**. The evolution of the reproductive strategy torwards a lower number of newborns for pregnancy, often accompanied by a higher level of maternal care, is the main reason for the large difference between NHP/Human and the other species.

Table 1. Anatomical features of the udders of the species taken into account.

Species	n° of glands	n° of teats/nipples	n° of canals per gland	Location	References
Rats	12	12	1	cervical (1pr) thoracic/pectoral (2pr) abdominal (1pr) inguinal (2pr)	[3, 4]
Mice	10	10	1	cervical (1pr) thoracic/pectoral (2pr) abdominal (1pr) inguinal (1pr)	[3, 5–8]
Rabbits	8-10	8-10	6-10	thoracic (1pr) abdominal (2pr) inguinal (1pr)	[9, 10]
Dogs	8-10	8-10	7-16	thoracic (2pr) abdominal/inguinal (2-3pr)	[8, 11]
(mini)Pigs	10-18	12-18	1-3	thoracic (2pr) abdominal (3pr) inguinal (2pr)	[12, 13]
NHPs	2	2	5-7	pectoral (1pr)	[14]
Humans	2	2	10-25	pectoral (1pr)	[3, 15]

Figure 1. Schematic representation of the anatomical features and distribution of the mammary gland in the analyzed species. Pink dots represent the canals/ducts. Artwork by Alberto Elmi.



In the analysed non-clinical species, at birth the gland is just a rudimentary ductal system, that will continue to evolve and grow during puberty and, mainly, first pregnancy under the influence of a wide variety of hormonal factors [2]. Indeed, out of the vast array of mammals' tissues, the mammary gland is one of the few undergoing multiple growth, functional development, and regression episodes in the lifespan. With the post-pubertal development, the area of the gland occupied by epithelium increases, with a relative decrease in its stromal component. Such phenomenon becomes even more evident in the late stages of gestation, when alveoli grow, even if, usually, true alveoli are not formed until conception. It still has to be acknowledged that the majority of critical changes (up to 94% of the overall modifications) occur during pregnancy [16]. During gestation, vascularization dramatically increases and, by mid-pregnancy, each alveolus is surrounded by a basket-like network of capillaries [17]. Milk secretion is achieved by the afore-mentioned alveoli, formed by a single layer of secretory epithelium bound by tight junctions and arranged in a cylindrical manner. Lobules, represented by multiple alveoli surrounded by connective tissue septa, generate lobes upon further bundling [18]. Another important component of the mammary gland is represented by myoepithelial cells, responsible for milk letdown, from alveoli down to the duct, and eventually milk release [18].

Humans

Humans have a single pair of mammary glands, called breasts, positioned over the *pectoralis major* muscle of the anterior chest. In humans, the mammary tissue is divided into 15-20 lobes of parenchyma separated from each other by a highly variable amount of adipose tissue. Each lobe is drained by its own major lactiferous duct leading to the nipple. The main ducts dilate into small sinuses as they get close to the *areolus*, where they open directly on the nipple. There are about 11 to 48 minor ducts. Surrounding the parenchymal structures are fibrous thickenings of connective tissue, which connect the deep fascia with the dermis of the overlying skin to form a suspensory ligament called Cooper's ligament. The functional Terminal Duct Lobular Unit (TDLU) appears in human breasts upon sexual maturity. According to the area occupied, number of acini, secretory morphology and cellularity, Lobular Units are classified as Types 1–4, with Type 1 being the least mature and Type 4 lobules being terminally differentiated, milk producing units found in the lactating mammary gland [3].

NHPs

Non-human primates, like humans, have two pectoral mammary glands. The non-lactating mammary gland is macroscopically flattened, but the histologic appearance is nearly identical to human breasts. In macaques, as in human women, the mammary tissue lies above and lateral to the nipple, extending to the axilla. The mature gland consists of an arboreous-like ductal system and TDLUs, which are formed of a terminal intralobular duct and surrounding alveoli, embraced by myoepithelial cells. In the

non-lactating breast, only approximately 5% of the organ is occupied by glandular epithelial tissue, while the remaining 95% consists of fat, fibrous connective tissue, and vascular and nervous structures. In NHPs, each nipple is crossed by five to seven lactiferous ducts, with varying degrees of communication between the corresponding ductal and lobular units. There are occasional small clusters of glandular tissue in the nipple [14].

(mini)Pigs

Out of the analysed species, the pig shows the highest variability in the number of mammary glands, mainly imputable by the wide range of breeds spread throughout the world. Breeds with higher number of teats have been selected by the farm industry as capable of nursing larger litter sizes, with higher economical profits. Generally speaking, pigs have six/seven pairs of mammary glands, located between the thoracic (two pairs), the abdominal (three pairs) and the inguinal (two pairs) area. Each nipple has two ducts which separately overlook on two external openings [18]. At birth, each mammary gland of the piglet is composed of the teat including its thick connective tissue base, an organized fat pad of adipose lobules and connective tissue, two lactiferous ducts, and a few ducts branching into the fat pad. These structures continue to grow until puberty. A significant increase in TDLU development occurs during pregnancy, particularly after day 75; during this period, parenchymal tissue mass increases by over 200%, while parenchymal lipid decreases by nearly 70% [19].

Dogs

Regarding the canine species, mammary glands are arranged into two lines along the ventral surface as for the other species, with two thoracic, one abdominal and two inguinal pairs. However, the number of the mammary glands in dogs can vary. Indeed, sometimes, the abdominal one is missing and occasionally there are more than five pairs. The adult mammary tissue is unevenly divided: the caudal glands are larger and the tissue of the two most caudal glands is usually continuous [11]. The amount of mammary and adipose tissue present is very variable and is more abundant in the abdominal and inguinal glands than in the thoracic glands. Each teat has between 7 and 16 duct openings, and each of these ducts will eventually form a lobe of the adult gland. The larger lactiferous ducts open into the mammary sinus that is lined by a double layer columnar epithelium, whereas the smaller ducts have a single layer of cuboidal epithelium and fusiform myoepithelial cells. Every duct is surrounded by fusiform myoepithelial cells. The alveolus is composed by secretory cells, which vary from cuboidal to columnar and have variable numbers of intracellular fat droplets that accumulate in the alveolar lumina. Surrounding the alveolus in a basket-like fashion, are star-shaped myoepithelial cells. The epithelial component of the mammary gland is supported by mesenchymal tissue; this

includes fibrous connective tissue, adipose tissue, blood vessels, nerves, and lymphatics. The fibrous connective tissue may be subdivided into 2 components: the intralobular component that surrounds the intralobular ducts and the interlobular component that separates the lobules. The first one consists of finer collagen fibers surrounded by a more extensive extracellular matrix, while the second has larger collagen fibers with less extracellular matrix [8].

Mice

Mice have five pairs of bilaterally symmetrical mammary glands, located along the ventral milk line between the cervical and inguinal area. Such lines can be divided into the cervical-thoracic area, containing three glands on each side, and the abdominal-inguinal region, with two glands on each side. Each gland terminates into a single collecting duct that releases milk through a single teat. Mice mammary glands, just like rats and rodents in general, do not have separate lobe and are made of a single complex arboreous system. Each rodent mammary gland contains 5-10 secondary collecting ducts, which drain into a single lactiferous duct in the nipple [3]. The mature secretory glandular unit is lobuloalveolar (LA), which undergoes complete maturation only during pregnancy and does not normally persist following weaning. Before pregnancy, ducts have blunt ends and will only develop terminal-end buds in pregnancy. The murine ductal system is primarily surrounded by adipose tissue, with poor fibrous tissue. Rodents mammary tissue comprehends two major epithelial cell types: basal myoepithelial cells and luminal epithelial cells. Luminal cells range from tall columnar cells in the major ducts to cuboidal cells in the smaller terminal ducts and lobules. Lobular luminal epithelial cells represent the actual functional secretory cell type during lactation [5].

Rats

Rats have twelve mammary glands, distributed in six pairs along the milk line, with one pair located in the cervical, two in the thoracic, one in abdominal and two in the inguinal regions. The organization of major lactiferous ducts is similar to the mouse, with a single duct leading to the nipple's ostia [3]. The mammary glands of females, comprised of scattered tubular ducts and alveolar structures, are characterized as tubuloalveolar. There are larger, more contiguous, lobular groups of cells distinguishable for their lack of tubular/ductal orientation [20]. The mammary gland has a compound of branching tubular ducts which terminate in secretory glandular alveoli, also called acini. Lobules are composed of groups of alveoli. As for the rat and the human species, the basic milk producing unit is the TDLU, composed of a lobule associated with intralobular and extralobular terminal ducts [3].

Rabbits

When compared to the other analysed species, data regarding the anatomy of the mammary gland in rabbits was lacking and very outdated. The number of mammary glands in this species can vary from 8 to 10 depending on the genetics of the animals [10]. They are distributed from the ventral thoracic to the inguinal regions: two pairs thoracic, two pairs abdominal and one pair inguinal. Each nipple has about 8-10 ostia [18].

Physiology of lactation

Lactation can be defined as the process that combines milk secretion and its removal and represents the final stage of the reproductive cycle. In order for it to be successful, 3 pivotal events have to occur: proliferation of alveolar epithelial cells, their structural and biochemical differentiation and, finally, synthesis and secretion of milk [18]. The process that leads to milk production is also known as lactogenesis and is critically linked to the acquisition of secretory capabilities by mammary alveolar cells. It is commonly divided into lactogenesis I and lactogenesis II [21]. During lactogenesis I, mammary epithelial cells (MECs) undergo morphological differentiation and become competent to produce and secrete some milk components referred to as colostrum [22]. In such phase, production of milk components seems to be restricted to a limited number of alveolar MEC as some secretory mechanisms are still incomplete [21]. During these late phases of pregnancy, milk production is blocked by the high levels of estrogens and, most importantly, progesterone, a steroid hormone also known as the “pregnancy hormone” since all mammals rely on this hormone to maintain pregnancy. Despite differences amongst species in progesterone production during pregnancy, a drastic drop in its production at parturition is always present [23]. Such drop allows for the initiation of the lactogenic complex activation and milk production, also referred to as lactogenesis II [18]. Indeed, high levels of progesterone are capable of inhibiting the most pivotal hormone related to lactation: prolactin (PRL). Its circulating concentrations slowly increase during pregnancy so that, by the end of gestation, levels are up to 20 times over pre-pregnancy reference values. Upon clearance of progesterone and estrogens at parturition, PRL can start promoting transcription of casein mRNA, stimulating the synthesis of α-lactalbumin, and increasing lipoprotein lipase activity in the mammary gland [24]. It is extremely important to acknowledge that PRL production, distribution, and its physiological functions are quite different in rodents when compared to humans and other mammals [25]. Once initiated, milk secretion continues but with variable rates over time [18], as is referred to as lactogenesis III [26]. Removal of the milk from the mammary gland is indeed necessary to maintain production and secretion. The overall control of milk secretion requires a strong interaction between both physical and chemical factors. Concerning physical factors, the most important one is the pressure exerted from the milk present in the alveoli that leads to an inverse relationship between milk production and

intra-mammary pressure. Indeed, as milk builds up within the mammary gland, crucial supporting structures such as blood vessels are displaced resulting in poor delivery of nutrients to the alveolar cells. Once milk is removed from the gland, pressure drops, and then slowly starts building up again as new milk is produced [18]. On the other hand, chemical control of milk production occurs locally by means of an autocrine protein fraction produced by MEC known as Feedback Inhibitor of Lactation (FIL) [21]. As of today, the exact mechanism of action of FIL is not completely clear, but it seems to be capable of slowing down milk production by suppressing key factors, stimulating intracellular breakdown of casein, reducing the number of PRL receptors, and inhibiting MEC differentiation [18]. Finally, another key hormonal factor in the lactation process is oxytocin. Suckling or manual stimulation of the teat is locally detected, and the stimulus is transmitted by sensory afferents to the hypothalamus, which then initiates oxytocin release from the neurohypophysis. This hormone stimulates the myoepithelial cells that surround the alveoli to contract and cause milk to flow from the alveoli through the duct system to the teat end [27].

The physiology of lactation does not differ drastically amongst species, but the duration and the yield of both colostrum and milk are highly variable (**Table 2**).

Table 2. Duration and yield of colostrum and milk production.

Species	Duration of colostrum production	Yield of colostrum	Duration of milk production	Yield of milk	Reference
Rats	/	/	~ 21 days	/	[28]
Mice	/	/	~ 18 days	0.1-0.5 ml	[29]
Rabbits	/	/	4-5 weeks	100-200 gr/day	[30, 31]
Dogs	48 h	270 ml/day	~ 8-10 weeks	~1000 ml/day	[32–35]
(mini)Pigs	24 h	~3.75k g/day*	~ 8 weeks	4500-5700 gr/day*	[36, 37]
NHPs	/	/	~ 12 months	/	[14, 38]
Humans	96 h	~500 ml/day	~ 6 months	~800 ml/day	[21, 39]

/ = data not available
* = these data only refer to standard pigs

Humans

Oxytocin, that slowly increases during late gestation and peaks at parturition, triggers milk ejection by inducing the contraction of myoepithelial cells and possibly by direct effects on the secretory activity of MEC; even bonding and maternal behaviors are regulated by oxytocin. In women, little to no milk can be obtained without activation of the milk ejection reflex (activation of both oxytocin and prolactin release). To be consistent with the duration of lactation in other primates, the average duration of

lactation in primitive women would be expected to be about 3 – 4 years [40], but nowadays the duration of breast-feeding in traditional highly industrialized societies varies greatly. It is impossible to determine 'normal' weaning behavior for both women and other mammals because the artificial termination of the lactation period is based either on social and cultural 'acceptability' or economic expediency [30].

NHPs

Non-human primate species have a high degree of similarities with humans. Oxytocin is low during the 3rd trimester of pregnancy, peaks on the date of parturition and returns to baseline levels during lactation [38]. Prolactin is not an obligate component of mammary growth and development in macaques but is required for lactation; this hormone is not as strong of a mitogen in the NHPs breast as steroid hormones or growth hormone (GH). In both human and non-human primates, the hepatic and intra-mammary enzymatic systems are present for conversion of precursor to a more bioactive estradiol (aromatase and steroid sulfatases); thus, the amount of local estrogen exposure in the breast correlates only weakly with the serum concentration. Gestation in macaques is approximately 150 days in length, and during this time, the breast, as in other mammals, undergoes extensive growth and differentiation under the influence of high systemic concentrations of estrogens, progestogens, chorionic gonadotropin, placental lactogen, and prolactin. The change in volume of the glandular tissue is roughly ten-fold to twenty-fold, as a result of both epithelial proliferation and secretory distention of the ductal and alveolar system [14].

(mini)Pigs

The peripartum prolactin (PRL) surge begins about 2 days prepartum and extends through several days postpartum, although it remains significantly greater than those found during most of pregnancy. The prepartum peak of PRL is essential for the onset of lactation and the decline slowly begins over the initial days postpartum [30]. In pigs and minipigs, the hormone relaxin from the corpora lutea has a similar function to placental lactogen, produced in humans and rodents, which is a prolactin agonist [23, 41]. Regarding the porcine species, most of the studies concerning lactation have been carried out under intensive breeding conditions and there is little information about pigs' lactation in wildlife. Wild sows build a nest for their litters and the piglets remain in the nests for about 2 weeks and are weaned after an 8-week lactation. In commercial intensive piggeries, the length of lactation has been truncated to 21-28 days (3-4 weeks), to increase profitability. The important role of milk ejection in lactation is clearly illustrated by the characteristic behavior pattern associated with suckling and the oxytocin release in the domestic sow, resembling what happens in humans. The piglets jostling and nuzzling on the mammary glands induce oxytocin release, followed by rapid ejection of milk from the

mammary glands. Piglets have a very short amount of time, in terms of minute, to obtain all the possible milk from their preferred nipple [30].

Dogs

Dogs also show a similar physiology of lactation with the drop in progesterone and estrogen post parturition, and increased levels of prolactin from mid gestation to weaning with higher spikes starting at parturition. Placental relaxin secretion starts from mid gestation, decreases in third trimester, and drops at parturition [42].

Mice

Although progesterone signalling controls alveolar proliferation, prolactin directly controls epithelial cell differentiation [43]. Its release is essential for the proliferation and functional differentiation of lobulo-alveolar structures during pregnancy [44]. Moreover, as opposed to humans, PRL has a strong luteotropic action in rodents, promoting progesterone production during pregnancy [25]. The estrogen hormone has receptors in both stromal and epithelial cells, but it is required only in the stroma for proper ductal development. Oxytocin, when released, induces the contraction of the myoepithelial cells surrounding the alveoli and thereby induces milk ejection. Thus, oxytocin is not only necessary for postpartum milk ejection but also for alveolar cell proliferation [43].

Rats

The physiology of rat lactation is similar to that reported for the mouse. In rats' mammary gland, PRLR expression is low during most of pregnancy and starts increasing on day 21, potentially in response to the pre-partum rise in pituitary PRL release, and continues to increase throughout lactation [45]. In female rats, lactation induces the mobilization of fat stores and a large increase in food intake, depending on the size of the suckling litter. In rats, prolactin secretion is suppressed during the second half of pregnancy [46]. The placenta produces the placental lactogen, which binds to prolactin receptors and stimulates growth and differentiation of epithelial cells in the udder in the same way as prolactin [41].

Rabbits

Data regarding the physiology of lactation in rabbits is unfortunately quite poor and outdated. What is known is that lactation usually lasts up to 4- 5 weeks [30], and that the litter only suckles one or twice a day. Also in this species, the key factor for lactation and its maintenance is prolactin [31].

Colostrum and milk composition

Milk and colostrum composition vary greatly among animal species. Components of milk and colostrum include proteins, lipids, carbohydrates, minerals, vitamins, and cells. The milk components were found to be influenced considerably by the stage of lactation, where these changes differ often from one species to another. In general, colostrum differentiates from milk mainly due to its high immunoglobulins (IgG) concentration. It is important to acknowledge that the different types of placentation and active immunity transport mechanisms, peculiar to each species, highly influence the degree of colostrum-mediated transfer of immunity. The degree and timing of immunity transfer during pregnancy to the offspring, impacts on the importance of colostrum for immunity transfer [18, 47]. In humans and NHPs, IgG are transferred to the fetus during the second and the third trimester of pregnancy while in rodents and rabbits IgG are transferred to the fetus mainly during organogenesis [47, 48]. In human and NHP most of the IgG are transferred to the fetus during pregnancy while in other mammalian species, such as the dog and the pig, IgG transfer during pregnancy is minimal hence the immunity transfer from the dam to the offspring is essentially lactogenic, with 85-95% of the blood immunoglobulins originating from colostrum transfer [49, 50]. Overall, IgG colostrum concentration is specifically high after parturition and rapidly drops. In addition to systemic immune protection, colostrum also plays a major role for local digestive system due to the presence of IgA, isoenzymes, lactoferrin, white blood cells and various cytokines. The newborn absorbs colostrum IgG from the digestive tract into the blood stream. The newborn ability to absorb IgG ends shortly after parturition [32]. While colostrum has higher concentrations of immunoglobulins in all mammalian species, the concentration of other components may vary between species. Very few papers investigating the milk composition early in lactation were found in our search. Here are shown the main characteristics and differences of the mammary secretion of the species examined for colostrum (**Table 3**) and “mature” milk (**Table 4**), respectively.

Table 3. Colostrum composition

Species	Dry matter %	Protein %	Casein %	Whey protein %	Fat %	Lactose %	Fe µg/ml	Cu µg/ml	Zn µg/ml	Mn µg/ml	Mg µg/ml	Ca µg/ml	P µg/ml	References
Rats	/	8.6-9.1	/	/	13.6-15.7	2.3-2.6	8.1-9.2	8.6-9.8	13.3-14.2	0.3-0.4	168-180	755-829	/	[51]
Mice	/	/	/	/	/	/	/	/	/	/	/	/	/	/
Rabbits	31.4-33.7	13.5-15.9	/	/	13.7-20.4	1.6-2.1	/	/	/	/	/	/	/	[52]
Dogs	/	12.4-16.2	60.7	39.3	13.2	1.7	3.7	1.3	5	/	128	1363	935	[53]
(mini)Pigs	20.1-26.7	7.7-16.6	1.5-3.4	7.9-14.8	6.4-8	2.8-3.9	/	/	/	/	100	800	1000	[37, 54]
NHPs	/	2.2-2.7	/	/	4.3-6.3	7.7-7.9	0.9-2.6	2-4.1	3.5-6.8	/	37.5-61.7	324-347	/	[55]
Humans	11.92	2.6	0.4	1.18	3	5.8	1.1	0.4	4.8	0.01	32	293	159	[56–58]

Data are expressed as ranges or single values.
/ = data not available

Table 4. Mature milk composition

Species	Dry matter %	Protein %	Casein %	Whey protein %	Fat %	Lactose %	Fe µg/ml	Cu µg/ml	Zn µg/ml	Mn µg/ml	Mg µg/ml	Ca µg/ml	P µg/ml	References
Rats	27.9-32.8	8.9-9.7	6.4-8	0.9-2.5	14-15.9	1.1-4.1	4-7	1.7-7	20333	/	158-192	2849-3206	1600-2720	[59, 60]
Mice	36.3-39.4	10.1-12.7	/	/	19.3-22.9	2.4-2.8	/	/	/	/	/	/	/	[29]
Rabbits	31.2	10.3	/	/	15.2	1.8	0.003§	0.002§	0.02-0.03§	0.0001§	0.35-0.45§	2.71-5.36§	2.44-3.28§	[52, 61]
Dogs	22.7-26	4.3-9.8	65.8-75.4*	26.4-34.2*	2.4-13.4	29.3-40.2	1.8-13.1	0.9-2	4.1-9.6	0.1-0.2	55.8-104.3	1366-2440	914-1401	[33, 53, 61–63]
(mini)Pigs	18.8-22.7	5-7.5	2.7-3.6	2.4-5.4	7-10.1	4.3-5.6	/	/	/	/	105	2000	1420	[37, 54]
NHPs	12.2-14	1.3-2.3	45*	55*	3.3-6.2	4.8-9.1	/	/	/	/	34	380	152	[61, 64–67]
Humans	12.6	1.2	0.3	0.7	4.1	7	0.5-1.8	0.2-5.2	0.7-3.8	0.01-0.03	25-33	230-310	130-190	[68–70]

Data are expressed as ranges or single values.
/ = data not available
* = % on total proteins
§ = these data are expressed in g/kg

Humans

As for the other mammals, the composition of human milk is affected by different factors and, depending on the individual, changes over the course of a single breast-feeding session, of a day, and through lactation [57, 69]. One of the key factors seems to be represented by the maternal diet: indeed dietary intake of different nutrients, particularly fatty acids and some micronutrients, is related to their content in breast milk composition, but does not affect macro-nutrient composition [71]. Generally speaking, fat concentrations tend to increase not only over the course of a day, but also during the same breastfeeding session [69]. On the other hand, the overall protein and amino acids contents show a marked decreasing trend over time during the first year of lactation [57]. One of the few components that seems to remain relatively stable for the entire duration of lactation, exception made for colostrum, is lactose [57]. Regarding minerals, magnesium, phosphorus and calcium show significant lactation-stage-specific differences and high inter-individual variations, while manganese, copper and iron remain relatively constant during all lactation stages. Zinc concentrations, overall, show a decreasing trend as lactation goes on [57].

NHPs

Non-human primate milk is relatively diluted: it generally consists of <15% dry matter, with about 7% sugar, ≅3–6% fat, and ≅1–2% proteins, and changes over the course of lactation. It is important to acknowledged that, amongst different NHPs species, great differences in milk composition can be observed, mainly imputable to length of lactation, frequency of feeding and milk yield [72, 73]. Most papers investigated the NHP milk composition used the rhesus macaque, while very few papers investigating the milk composition of cynomolgus monkeys, which is the most common NHP in research, were found. In rhesus macaque (*Macaca mulatta*) both fat and protein contents increase as infants age, in the light of the higher demand in energy. Such increase in energy content seems to be related to lower milk yields. Out of the different components, fat percentages show the highest inter individual variation within the same species, while lactose levels are relatively stable [66]. However, differences in milk composition among prosimians may be related to differences in maternal care: species that carry their offspring produce more dilute milk, with higher yields, when compared to species that usually leave newborns for prolonged period. Lorises, bushbabies, and potentially cheirogaleids produce relatively rich, energy-dense milks in comparison with anthropoid primates, such as rhesus macaques (*Macaca mulatta*), white handed gibbon (*Hylobates lar*) and gorilla (*Gorilla gorilla gorilla*) [72].

(mini)Pigs

When compared to mature milk, colostrum has higher concentrations of protein, particularly

immunoglobulins, some minerals (particularly copper, iron, iodine, and zinc) and vitamins, hormones and growth factors. Lactose is present in lower concentrations in colostrum than in mature milk. Milk fat concentration transiently increases during the period from day 2 to day 4. The composition of milk after approximately day 7 to day 10 is relatively stable for the remainder of lactation. As for other species, maternal diet can affect some milk components, including concentrations of fat, fat-soluble vitamins and some minerals, as well as proportions of specific fatty acids. Some components of sow milk also are affected by genetics, parity, colostrum and milk yield, and ambient temperature [37].

Dogs

Early studies showed the dog milk composition might change with breed. For this summary, we focused on the beagle dog as it is the commonly used breed for research purposes.

To summarize, the concentration of iron, zinc, calcium, protein and fat showed patterns that were influenced by the stage of lactation. The concentration of copper, manganese, magnesium and carbohydrates were not significantly affected.

Adkins et al found that protein concentration was high in colostrum, decreased significantly by Day 21, and then slighty increased throughout the duration of lactation. This pattern of decrease protein concentration during lactation is similar to humans [53]. However, Lonnerdal et al reported that protein concentration increased in time [62]. Concentration of amino acid content were similar to those in humans. A decrease pattern in the concentration of all amino acids with increasing lactation stage was observed, similar to humans. The lipid content does not show remarkable changes during the lactation period. Slight non-significant decreases were observed between days 14 to 28. The lipid concentration in dog milk was higher than that reported in humans [53]. Lactose levels were low in colostrum, increasing gradually until Day 28, followed by a slight decrease. Iron concentration increased significantly from Day 1 to Day 3, then gradually decreased by Day 42. This is in contrast to humans where iron concentration is high in the colostrum and then gradually decrease during lactation [53]. Lonnerdal et al reported that Zinc concentration decreased throughout lactation [62] while Adkins et al reported that zinc concentration slowly increased from Day 1 to Day 14 and it then decreased by Day 42. Zinc levels were higher than those reported for humans [53, 62]. Copper concentration was slightly higher in early lactation and then gradually decreased throughout lactation or remained unchanged. Copper levels were generally higher than reported in humans. Milk calcium concentration was lower in colostrum but increased thereafter peaking on Day 35. Calcium levels were significantly higher than reported for humans [53, 62]. Magnesium concentration was highest in colostrum but rapidly decreased by Day 3 and remained relatively constant during lactation. Concentration of phosphorus showed very mild increase from Day 3 to Day 28 [53]. The iron concentration in the dog milk are influenced by the stage of lactation, with values decreasing in time. This is similar to what was reported in other species however, the iron concentration in dog milk was

found to be considerably higher than that of human milk. The manganese concentration was not found to be influenced by the stage of lactations and its levels were higher than reported for human milk. The fat content of canine milk was found to be influenced by the stage of lactation, with concentrations increasing during the first part of lactation and decreasing during the last part [62]. The level of carbohydrates was fairly constant and did not show a strong developmental pattern.

Mice

Very few papers were published on mouse milk composition. The composition of mouse milk can vary considerably between mouse strains. In general, the analysis on mouse milk is challenging due to the small sample volume and the high fat content. Crude protein levels did not change during lactation [29, 74, 75]. Crude fat increased from Day 3 of lactation to Day 14 and remain stable till Day 18 [29, 74], while in another study the fat content decreased from early to mid-lactation [76]. Lactose content increased with lactation day [29, 75]. Great variability was noted in lactose content and crude protein between strains of mice.

Rats

There are characteristic differences in the nutrient content of milk among strains of rats, particularly for changes in the lactose and fat content of the milk during the lactation period. In general, rat milk elements (iron, copper, zinc, manganese) show a similar pattern of high initial levels, mainly during the colostrum phase, that decrease throughout lactation. The concentration of some elements increases during the last phase of lactation (iron, manganese). The iron concentration of rat milk is considerably higher than that found in human milk, and is much greater than the rat plasma iron levels. The concentrations of iron in rat milk decrease rapidly during the first part of lactation (approx. 40% drop). Thereafter, the concentration of iron continues to decrease but in a less pronounced manner. In the last days of lactation (Days 25-28), the milk iron increases. A decrease in iron content with lactation time was also found in humans, although the percentage decrease is not that high. Colostrum copper concentration are considerably higher than that of humans and decreases during the first 7 days of lactation. Copper concentration continues to decrease until Day 11, but to a lesser extent, and then remains relatively unchanged to the end of lactation. The change in copper concentration pattern is similar to that observed in humans, however copper concentration in rat is much higher. Zinc concentration is high during the colostrum period and decreases significantly during the course of lactation. Similar to copper, the change in zinc concentration pattern is similar to that observed in humans, however zinc concentration in rat is much higher. The concentration of manganese decreases significantly from Day 0 to Day 12 and remains low, but increases to nearly initial levels at the last days of lactation. Similar pattern of manganese concentration was reported in

humans. In contrast to other elements, the concentration of manganese in rat milk is not considerably higher than that of humans. Magnesium concentration was fairly stable during early and mid-lactation, but decreases during late lactation. The protein and calcium concentration increase steadily during lactation till day 24 and decreases at the end of the lactation period. The similar patterns of calcium and protein is likely due to the fact that the major protein of rat milk is casein, which is well known for its calcium binding capability. In contrast to rats, human calcium levels decline during lactation. Carbohydrate concentration increases during the first half of lactation, then decreases during the second half. In humans' milk carbohydrates level is much higher than in rats. In humans an increase in milk carbohydrate is also found in the early lactation period, but there is no decrease at later stages of lactation. While lactose is by far the major carbohydrate in human milk, rat milk may contain significant amounts of neuraminlactose. Quantitatively, the most important constituent in rat milk is fat. Similar to humans, the fat content of the rat milk did not exhibit a strong pattern during the lactation period [51].

Rabbits

Kits are weaned at the age of 4-5 weeks and are exclusively dependent on milk until lactation Day 18-19. Rabbit milk yield corresponds to kits weaning stage and reaches its peak around lactation days 17-21. It is important to remember that rabbits only nurse kits once or twice a day, strongly impacting the nutritional value of the milk. In general, rabbits' milk is concentrated with fat, protein and energy but nearly absent of lactose, and the composition does not vary significantly between most breeds. As in other mammalian species, the colostrum has higher protein content due to high immunoglobulin level. This increases the dry matter value of colostrum relative to rabbit milk. Protein content decreases along with the increase of milk yield during lactation. Apart from protein content, the composition of rabbit milk is quite stable during the 2nd and 3rd week of lactation. The changes in composition in the later stage of lactation are closely related with decrease in milk yield. Mineral element composition changes substantially after lactation peak. In general, rabbit milk is reach with calcium, sodium and potassium. Calcium concentration and to a lesser extent phosphorus increases with progressing lactation stage, while the effect on potassium and sodium is less clear and there are different data in different papers. Magnesium content increases with lactation stage while zinc, copper, iron and manganese decrease gradually in concentration as lactation progresses [52].

Discussion

Anatomy of the mammary gland

As expected, from a gross anatomy point of view, the variations between the analysed species are very high. When looking at the number and the position of glands, NHPs better resemble the human

situation, with differences only in the ducts. The number of teats is directly related to the number of the offspring [16], therefore such situation was to be expected. Pigs are on the opposite side of the spectrum as, depending on the breed and in light of the pork production requests, sows can deliver up to 18 piglets. Regarding canals/ducts, the human mammary gland is the one with the highest number out of the species taken into account, followed by dogs. A peculiar situation can be noted in rodents, were only 1 canal per teat is present. When selecting a relevant animal model for trials involving lactation, gross anatomy is not necessarily one of the key decisional factors. Indeed, having a higher number of mammary glands and teats, often allows for easier sampling procedures and higher volume specimens. From a logistics perspective, it would be indeed easy to collect samples from one teat without disturbing the animal in the feeding process if more teats are available.

Physiology of lactation

No major discrepancies were noticed regarding the physiology of lactation amongst the analysed animals. Indeed, hormonal inputs and pathways are pretty much conserved, with prolactin and oxytocin being pivotal. Nonetheless, PRL seems to play different roles in rodents when compared to the other species analysed in the present literature scanning. When looking at the most important veterinary textbooks for such topic, the bovine species is always the most representative since its role in the milk industry for human consumption is undeniable. This represents a relative limitation for experimental animals, and in particular laboratory animals, since the peculiar gastrointestinal apparatus of ruminants changes the metabolic scenario of milk production. Several knowledge gaps need to be filled regarding physiology of lactation, both at systemic and molecular levels, including in—depth characterization of hormonal mechanisms of action and environmental influences among the others.

Colostrum and milk composition

The comparison of colostrum/milk compositions between the different species can be hard to interpret in light of several technical and physiological issues. Indeed, a lot of the retrieved papers are relatively old and rely on poor sample sizes and different, often outdated, analytical tools. Moreover, high interindividual variations, mainly qualitative, within the same species are extremely common and depend on a wide variety of factors including maternal nutritional status and diet, number of newborns, or duration of lactation. Colostrum of all species contains high levels of IgG and other immune factors which increase the protein content. The level of IgG decreases within a few days post partum. As for the other components of both milk and colostrum, they vary between species and the NHPs seem to better resemble humans' mammary gland secretions, definitely more diluted when compared to the other species like rabbits, rats and mice. This finding was to be expected since, as already mentioned, the number of newborns and maternal care are amongst the key factors. Nonetheless, also (mini)pigs

show a relatively close similarity with humans' milk gross composition despite their capability to produce high litter sizes.

Practical Considerations

Choosing an animal model for a particular trial is always difficult and often implies the necessity to cope with knowledge and literature gaps. The topics hereby covered represent the starting point for a conscious decision, since anatomical and physiological similarities to humans are the basis to a high translational value experimentation. Looking at the results, NHPs, as expected, seem to represent the best model to enrol in studies regarding lactation. Nonetheless, other factors have to be considered, two of the most important being the ethical and the economical one. In the last decade, the scientific community has come to the agreement of using experimental NHPs only when strictly necessary [77]. Moreover, trials involving NHPs are expensive, long, and due to ethical considerations have low sample size that can undermine the outcome data. Finally, collecting milk samples from such species could be extremely difficult in light of the maternal behaviour. Ease of samples collection should always be a key factor when designing animal trials, as choosing a "difficult" species may lead to failure especially in "longer" trials that require un-sedated animals and/or repeated samples. Smaller animals such as rodents and rabbit are not necessarily the best choice for lactation studies. Indeed, in order to collect milk from rodents, one of the few options is the euthanasia of pups to collect gastric content right after suckling. Other methods, such as using mini-pumps to collect milk directly from the mother, often require hormonal injection to increase milk volume, a strong bias in drug lactation transfer studies, and still lead to small volume of samples. Furthermore, their milk composition resulted quite different form humans' one, and discrepancies in PRL production and functions were highlighted. In such scenario, larger animals like dogs and pigs seem to find a good fit for lactation trials. With regards to dogs, it is important to acknowledge that, despite their relevance for regulatory toxicology studies, their use for biomedical research often raises strong criticism and ethical issues in public opinion, especially in Europe. On the other hand, the enrolment of pigs in research trials seems to be more widely accepted and is considered as a valid alternative when anatomical/physiological differences are not relevant [78]. Minipigs in particular offer all the advantages of conventional pigs including genetic and metabolic similarities to humans, avoiding the main problem represented by the size. Their use in the biomedical setting is well established and recognized, and the availability of in depth physiological characterization and the related physiology-based models, vital for results interpretation, is increasing. Moreover, Göttingen Minipigs are specifically produce for biomedical purposes, with high standardized genetic background and health status.

Conclusion

In conclusion, the present analysis of the literature confirmed the complexity of the decisional process behind the choice of an animal model for *in vivo* trials. For some of the scanned species, data were either poor or completely missing, highlighting the necessity to generate more physiological background studies for species that are routinely used in laboratory settings.

Overall, the Göttingen minipig seems to represent the better choice when looking at both physiological similarities with humans and feasibility of lactation trials.

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Review

Animal Models for In Vivo Lactation Studies: Anatomy, Physiology and Milk Compositions in the Most Used Non-Clinical Species: A Contribution from the ConcePTION Project

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Simple Summary: Nowadays, the importance of breastfeeding has been very well recognized not only by the scientific world but also by public opinion. Such awareness has nonetheless put a lot of pressure on women under chronic pharmacological medication, or that simply need to alleviate common post-partum health issues, due to the lack of scientific data regarding the potential transfer to the offspring during lactation. In such a scenario, the ConcePTION project aims at creating a trusted ecosystem that can efficiently generate and disseminate reliable evidence-based information regarding the effects of medications used during pregnancy and breastfeeding to women and their healthcare providers. Due to the need for a reliable animal species to obtain scientific data, the present review summarizes the main features contributing to the lactation process in the most commonly used laboratory animal species.

Abstract: The present review aims to summarize the main features of mammary gland anatomy, and the physiology of lactation and colostrum/milk in the most commonly used animal species for regulatory toxicity. The final goal is the selection of a preferred animal species to be enrolled in studies investigating the potential transfer of drugs and exogenous molecules through milk, within the Innovative Medicines Initiative (IMI) funded project ConcePTION. Reference data regarding humans were also collected and analyzed in order to highlight critical similarities and differences with the studied species. Additional practical considerations were also taken into account, such as ethical consideration regarding the chosen species which affects the group size, financial implications and technical feasibility of lactation trials (e.g., ease of sampling, volume of sampling, husbandry requirements and scientific recognition). In conclusion, the present analysis of the literature confirms the complexity of the decisional process behind the choice of an animal model for in vivo trials. For some of the evaluated species, data were either poor or missing, highlighting the necessity to generate more physiological background studies for species that are routinely used in laboratory

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settings. Overall, when taking into consideration ethical factors, feasible group size, milk volume and ease of milk collection, and physiological similarities with humans, minipigs seem to represent the most appropriate choice.

Keywords: animal models; mammary gland; lactation; milk; colostrum; mice; rats; rabbits; dogs; non-human primates; pigs; minipigs; human

1. Introduction

As of today, the importance of breastfeeding has been very well recognized not only by the scientific world but also by public opinion. Indeed, colostrum and breast milk represent the gold standard when it comes to nutritional and protective values [1], as also recommended by the World Health Organization [2]. Such awareness has nonetheless put a lot of pressure on women under chronic pharmacological medication, or that simply need to alleviate common post-partum health issues such as infections, mastitis, and headaches [3]. Indeed, the lack of reliable evidence-based knowledge regarding the safety of medications during lactation often leads physicians and medical practitioners to advise women to stop breastfeeding [4,5]. Behind the lack of scientific data are a plethora of different reasons, including the absence of a recognized "state of the art" animal model for preclinical studies aimed at testing the potential transfer through milk of systemically administered exogenous compounds such as drugs and medications.

Choosing the best animal model for a given experiment can be challenging and represents a complex decisional process that should take into account a wide variety of factors [6]. Generally speaking, the decision should be made upon in-depth consideration of: (i) analogy/homology; (ii) translation value to humans; (iii) genetic standardization; (iv) biological background knowledge; (v) cost/availability; and (vi) adaptability to experimental procedures [7]. For lactation studies, the key features to be analyzed are anatomy of the mammary gland, physiology of lactation, and colostrum/milk composition. Due to the pivotal role played by gastrointestinal/energetic metabolism in every biological process, ruminants should be used very cautiously when selecting an animal model for metabolism-related studies. Indeed, their peculiar gastrointestinal anatomy and physiology results in a relatively lower translational value when (drug) metabolism is involved, thus creating strong biases when considering the overall aim of such trials.

Literature searches were performed to identify a candidate non-clinical species relevant to humans for use in lactation studies and experimental trials. Species taken into account were the most commonly used in regulatory toxicology and included rodents (rat and mice), rabbits, dogs, pigs (both conventional and minipigs) and non-human primates (NHPs) [8]. Reference data regarding humans were also collected and analyzed in order to highlight critical similarities and differences with the studied species.

A preliminary scanning of the literature highlighted a relative lack of relevant data in the most used databases such as PubMED and EMBASE, making a systematic approach unfeasible. Indeed, basic data regarding anatomy and physiology are often found in textbooks or old papers and used only as reference/control data in more recent studies. Therefore, it was decided to broaden the literature search to currently used veterinary medicine textbooks, and to use less-specific search words and their combination for the different subcategories. Further literature searches were then performed starting from the reference sections of the retrieved articles.

The search criteria are provided below.

Anatomy of the mammary gland: preliminary search words included ("mammary gland" OR "udder") AND ("anatomy" OR "morphology" OR "structure") AND the aforementioned different species and their synonyms.

Physiology of lactation: preliminary search words included ("lactation" OR "milk production" OR "colostrum production") AND ("physiology") AND the aforementioned different species and their synonyms.

Colostrum/milk composition: preliminary search words included ("colostrum" OR "milk") AND ("composition" OR "components" OR "quantitative composition") AND the aforementioned different species and their synonyms.

Retrieved articles, book chapters and books were then analyzed to identify potential biases to the reported results as methodological errors or experimental design errors.

2. Anatomy of the Mammary Glands

In general, mammary glands can be defined as modified glands that give name to the Mammalia class, whose secretion is vital for offspring survival. They are complex tubule-alveolar glands made of secretory units organized into lobules, surrounded by connective tissue septa [9]. From a developmental point of view, they originate as epithelial buds growing into the mesenchyme starting from linear ectodermal thickenings, also known as mammary ridges, and subsequently placodes [10]. Mesenchyme starts proliferating around such buds to create a teat/nipple on the skin surface. At this stage, epidermal sprouts start developing, from the buds to the teat/nipple, creating canals. Every canal will then create a separate duct that associates with a glandular mass and a separate orifice [9]. The number of overall glands, teats/nipples and canals vary amongst mammals as shown in Table 1, as well as the anatomical location of the mammary unit, represented in Figure 1. The evolution of the reproductive strategy towards a lower number of newborns, often accompanied by a higher level of maternal care, is the main reason for the large difference between NHP/humans and the other species.

Table 1. Anatomical features of the udders of the species taken into account; pr = per row.

Species	Number of Glands	Number of Teats/Nipples	Number of Canals per Gland	Location	References
Rats	12	12	1	cervical (1 pr) thoracic/pectoral (2 pr) abdominal (1 pr) inguinal (2 pr)	[11,12]
Mice	10	10	1	cervical (1 pr) thoracic/pectoral (2 pr) abdominal (1 pr) inguinal (1 pr)	[11,13–16]
Rabbits	8–10	8–10	6–10	thoracic (1 pr) abdominal (2 pr) inguinal (1 pr)	[17,18]
Dogs	8–10	8–10	7–16	thoracic (2 pr) abdominal/inguinal (2–3 pr)	[16,19]
(Mini)Pigs	10–18	12–18	1–3	thoracic (2 pr) abdominal (3 pr) inguinal (2 pr)	[20,21]
Non-Human Primates (NHPs)	2	2	5–7	pectoral (1 pr)	[22]
Humans	2	2	10–25	pectoral (1 pr)	[11,23]

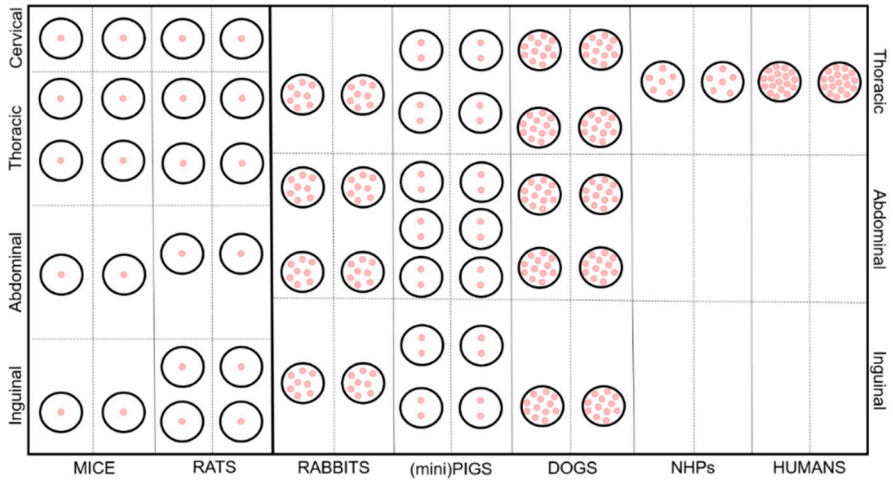

Figure 1. Schematic representation of the anatomical features and distribution of the mammary gland in the analyzed species. Pink dots represent the canals/ducts.

In the analyzed non-clinical species, at birth the gland is just a rudimentary ductal system, that will continue to evolve and grow during puberty and first pregnancy/lactation, under the influence of a wide variety of hormonal factors [10]. As for many developmental patterns, the exact timing of this process varies depending on the mammalian species taken into consideration. As a matter of fact, out of the vast array of mammal tissues, the mammary gland is one of the few undergoing multiple growth, functional development, and regression episodes in the lifespan. With the post-pubertal development, the area of the gland occupied by epithelium increases, with a relative decrease in its stromal component. Such phenomena become even more evident in the late stages of gestation, when alveoli grow, even if, usually, true alveoli are not formed until conception. Generally speaking, it still has to be acknowledged that the majority of critical changes, despite some species-specific differences, occur during pregnancy [10,24]. During gestation, vascularization dramatically increases, and by mid-pregnancy, each alveolus is surrounded by a basket-like network of capillaries [25]. Milk secretion is achieved by the aforementioned alveoli, formed by a single layer of secretory epithelium bound by tight junctions and arranged in a cylindrical manner. Lobules, represented by multiple alveoli surrounded by connective tissue septa, generate lobes upon further bundling [24]. From a histological point of view, mammary glands can indeed be generally described as an epithelial bilayer, within adipose tissue, with two key cell populations: luminal cuboidal cells and star-shaped myoepithelial cells [26,27]. The first population lines the lactiferous ducts, form the inner portion, while myoepithelial cells constitute the outer portion of the bilayer and are responsible for milk ejection [28].

2.1. Humans

Humans have a single pair of mammary glands, called breasts, positioned over the *pectoralis major* muscle of the anterior chest. In humans, the mammary tissue is divided into 15–20 lobes of parenchyma separated from each other by a highly variable amount of adipose tissue. Each lobe is drained by its own major lactiferous duct leading to the nipple. The main ducts dilate into small sinuses the closer they are to the *areolus*, where they open directly on the nipple. There are about 11 to 48 minor ducts. It is nonetheless important to acknowledge that the presence of sinuses in the human mammary gland is matter of debate, as pointed out by the work of Ramsay et al. [29]. Surrounding the parenchymal structures are fibrous thickenings of connective tissue, which connect the deep fascia with the dermis of the overlying skin to form a suspensory ligament called Cooper's ligament. The functional terminal duct lobular unit (TDLU) appears in human breasts upon sexual maturity. According to the area occupied, number of acini, secretory morphology and cellularity, lobular units are classified as Types 1–4, with Type 1 being the least mature and

Type 4 lobules being terminally differentiated, milk-producing units found in the lactating mammary gland [11,30].

2.2. NHPs

Non-human primates, similarly to humans, have two pectoral mammary glands. The non-lactating mammary gland is macroscopically flattened, but the histologic appearance is nearly identical to human breasts. In macaques, as in women, the mammary tissue lies above and lateral to the nipple, extending to the axilla. The mature gland consists of an arboreous-like ductal system and TDLUs, which are formed of a terminal intralobular duct and surrounding alveoli, embraced by myoepithelial cells. In the non-lactating breast, only approximately 5% of the organ is occupied by glandular epithelial tissue, while the remaining 95% consists of fat, fibrous connective tissue, and vascular and nervous structures. In NHPs, each nipple is crossed by five to seven lactiferous ducts, with varying degrees of communication between the corresponding ductal and lobular units. There are occasional small clusters of glandular tissue in the nipple [22].

2.3. Pigs and Minipigs

Out of the analyzed species, the pig shows the highest variability in the number of mammary glands, mainly imputable by the wide range of breeds spread throughout the world. Breeds with higher number of teats have been selected by the farm industry as capable of nursing larger litter sizes, with higher economic profits. Generally speaking, pigs have six/seven pairs of mammary glands, located between the thoracic (two pairs), the abdominal (three pairs) and the inguinal (two pairs) area. Each nipple has two ducts which separately lead to two external openings [24]. At birth, each mammary gland of the piglet is composed of the teat including its thick connective tissue base, an organized fat pad of adipose lobules and connective tissue, two lactiferous ducts, and a few ducts branching into the fat pad. These structures continue to grow until puberty. A significant increase in TDLU development occurs during pregnancy, particularly after day 75; during this period, parenchymal tissue mass increases by over 200%, while parenchymal lipid decreases by nearly 70% [31]. In such a scenario, prolactin has been proven to be responsible for growth and differentiation on porcine mammary epithelium, with late gestational hyperprolactinemia leading to enhanced lactogenesis and milk production without altering vascular development [32,33].

2.4. Dogs

Regarding the canine species, mammary glands are arranged into two lines along the ventral surface as for the other species, with two thoracic, one abdominal, and two inguinal pairs. However, the number of the mammary glands in dogs can vary. Indeed, sometimes, the abdominal pair are missing, and occasionally there are more than five pairs. The adult mammary tissue is unevenly divided: the caudal glands are larger and the tissue of the two most caudal glands is usually continuous [19]. The amount of mammary and adipose tissue present is very variable and is more abundant in the abdominal and inguinal glands than in the thoracic glands. Each teat has between 7 and 16 duct openings, and each of these ducts will eventually form a lobe of the adult gland. The epithelial component of the mammary gland is supported by mesenchymal tissue; this includes fibrous connective tissue, adipose tissue, blood vessels, nerves, and lymphatics. As in humans, the fibrous connective tissue may be subdivided into two components: the intralobular component that surrounds the intralobular ducts, and the interlobular component that separates the lobules. The former consists of finer collagen fibers surrounded by a more extensive extracellular matrix, while the latter has larger collagen fibers with less of an extracellular matrix [16]. In dogs, the involution of the mammary glands starts around the eighth week of lactation, and progresses until the end of the third month [34].

2.5. Mice

Mice have five pairs of bilaterally symmetrical mammary glands, located along the ventral milk line between the cervical and inguinal area. Such lines can be divided into the cervical–thoracic area, containing three glands on each side, and the abdominal–inguinal region, with two glands on each side. Each gland terminates into a single collecting duct that releases milk through a single teat. Mouse mammary glands, just like rats and rodents in general, do not have separate lobes and are made of a single complex arboreous system. Each rodent mammary gland contains 5–10 secondary collecting ducts, which drain into a single lactiferous duct in the nipple [11]. The mature secretory glandular unit is lobuloalveolar (LA), which undergoes complete maturation only during pregnancy and does not normally persist following weaning. During puberty, terminal end buds start forming, directing ductal elongation. The murine ductal system is primarily surrounded by adipose tissue, with poor fibrous tissue.

2.6. Rats

Rats have twelve mammary glands, distributed in six pairs along the milk line, with one pair located in the cervical, two in the thoracic, one in abdominal and two in the inguinal regions. The organization of major lactiferous ducts is similar to the mouse, with a single duct leading to the nipple's ostia [11]. The mammary glands of females, comprising scattered tubular ducts and alveolar structures, are characterized as tubuloalveolar. There are larger, more contiguous, lobular groups of cells distinguishable for their lack of tubular/ductal orientation [35]. The mammary gland has a compound of branching tubular ducts which terminate in secretory glandular alveoli, also called acini. Lobules are composed of groups of alveoli. As for the rat and the human species, the basic milk-producing unit is the TDLU, composed of a lobule associated with intralobular and extralobular terminal ducts [11].

2.7. Rabbits

The number of mammary glands in this species can vary from 8 to 10 depending on the genetics of the animals [18], with approximately 6–7 ductal systems per gland [36]. They are distributed from the ventral thoracic to the inguinal regions: two pairs of thoracic, two pairs of abdominal and one pair of inguinal mammary glands. Each nipple has about 8–10 ostia [24]. The presence of sinus-like dilatation of the ducts has been described in pregnant/lactating European breeds, seemingly acting as a milk reservoir [36]. In this species, the majority of the mammary developmental process takes place during gestation (approximately 67%), with the remaining 33% occurring during lactation [37,38]. It is nonetheless important to mention that growth dynamics seems to be influenced both by breed, husbandry conditions, and experimental methodologies employed [37].

3. Physiology of Lactation

Lactation can be defined as the process that combines milk secretion and its removal and represents the final stage of the reproductive cycle. In order for it to be successful, three pivotal events have to occur: proliferation of alveolar epithelial cells, their structural and biochemical differentiation and, finally, synthesis and secretion of milk [24]. The process that leads to milk production is also known as lactogenesis and is critically linked to the acquisition of secretory capabilities by mammary alveolar cells. It is commonly divided into lactogenesis I, II and III [39]. Lactogenesis I is also referred to as "secretory differentiation", while lactogenesis II is "secretory activation" [40]. During lactogenesis I, mammary epithelial cells (MECs) undergo morphological differentiation and become competent to produce and secrete some milk components referred to as colostrum [41]. In such phases, production of milk components seems to be restricted to a limited number of alveolar MEC because some secretory mechanisms are still incomplete [39]. During these late phases of pregnancy, milk production is blocked by the high levels of estrogens and, most importantly, progesterone, a steroid hormone also known as the "pregnancy

hormone" because all mammals rely on this hormone to maintain pregnancy. Despite differences amongst species in progesterone production during pregnancy, a drastic drop in its production at parturition is always present [42]. Such a drop allows for the initiation of the lactogenic complex activation and milk production, also referred to as lactogenesis II [24]. Indeed, high levels of progesterone are capable of inhibiting the most pivotal hormone related to lactation: prolactin (PRL). Its circulating concentrations slowly increase during pregnancy so that, by the end of gestation, levels are up to 20-fold higher than pre-pregnancy reference values. Upon the clearance of progesterone and estrogens at parturition, PRL can start promoting the transcription of casein mRNA, stimulating the synthesis of α-lactalbumin, and increasing lipoprotein lipase activity in the mammary gland [43]. It is extremely important to acknowledge that PRL production, distribution, and its physiological functions are quite different in rodents when compared to humans and other mammals [44]. Once initiated, milk secretion continues but with variable rates over time [24], as is referred to as lactogenesis III [45]. Removal of the milk from the mammary gland is necessary to maintain its production and secretion. The overall control of milk secretion requires a strong interaction between both physical and chemical factors. Concerning physical factors, most important is the pressure exerted from the milk present in the alveoli that leads to an inverse relationship between milk production and intra-mammary pressure. As milk builds up within the mammary gland, crucial supporting structures such as blood vessels are displaced, resulting in poor delivery of nutrients to the alveolar cells. Once milk is removed from the gland, pressure drops, and then slowly starts building up again as new milk is produced [24]. On the other hand, chemical control of milk production occurs locally by means of an autocrine protein fraction produced by MEC known as the feedback inhibitor of lactation (FIL) [39]. Currently, the exact mechanism of action of FIL is not completely clear, but it seems to be capable of slowing down milk production by suppressing key factors, stimulating the intracellular breakdown of casein, reducing the number of PRL receptors, and inhibiting MEC differentiation [24]. Finally, another key hormonal factor in the lactation process is oxytocin. Suckling or manual stimulation of the teat is locally detected, and the stimulus is transmitted by sensory afferents to the hypothalamus, which then initiates oxytocin release from the neurohypophysis. This hormone stimulates the myoepithelial cells that surround the alveoli to contract and cause milk to flow from the alveoli through the duct system to the teat end [28,46]. The physiology of lactation does not differ drastically amongst species, but the duration and the yield of both colostrum and milk are highly variable (Table 2).

Table 2. Duration and yield of colostrum and milk production.

Species	Duration of Colostrum Production	Yield of Colostrum	Duration of Milk Production	Yield of Milk	References
Rats	/	/	~21 days	/	[47]
Mice	/	/	~18 days	0.1–0.5 mL	[48]
Rabbits	/	/	4–5 weeks	100–200 g/day	[49,50]
Dogs	48 h	270 mL/day	~8–10 weeks	~1000 mL/day	[51–54]
(mini)Pigs	24 h	~3.75 kg/day *	~8 weeks	4500–5700 g/day *	[55,56]
NHPs	/	/	~12 months	/	[22,57]
Humans	96 h	~500 mL/day	~6 months	~800 mL/day	[39,58]

/, data not available; *, these data only refer to standard pigs.

3.1. Humans

Oxytocin, which slowly increases during late gestation and peaks at parturition, triggers milk ejection by inducing the contraction of myoepithelial cells and possibly, by direct effects on the secretory activity of MEC, even bonding and maternal behaviors are regulated by oxytocin. In women, little to no milk can be obtained without activation of

the milk ejection reflex (activation of both oxytocin and PRL release). To be consistent with the duration of lactation in other primates, the average duration of lactation in women in ancient times would be expected to be about 3–4 years [59], but nowadays the duration of breast-feeding in traditional highly industrialized societies varies greatly. It is impossible to determine "normal" weaning behavior for both women and other mammals because the artificial termination of the lactation period is based either on social and cultural "acceptability" or economic expediency [49].

3.2. NHPs

Non-human primate species have a high degree of similarities with humans. Oxytocin is low during the third trimester of pregnancy, peaks on the date of parturition, and returns to baseline levels during lactation [57]. Prolactin is not an obligate component of mammary growth and development in macaques but is required for lactation; this hormone is not as strong of a mitogen in the NHP breast as steroid hormones or growth hormone (GH). In both human and non-human primates, the hepatic and intra-mammary enzymatic systems are present for the conversion of precursors to a more bioactive estradiol (aromatase and steroid sulfatases); thus, the amount of local estrogen exposure in the breast correlates only weakly with the serum concentration. Gestation in macaques is approximately 150 days in length, and during this time, the breast, as in other mammals, undergoes extensive growth and differentiation under the influence of high systemic concentrations of estrogens, progestogens, chorionic gonadotropin, placental lactogen, and PRL. The change in volume of the glandular tissue is roughly ten-fold to twenty-fold, as a result of both epithelial proliferation and secretory distention of the ductal and alveolar system [22].

3.3. Pigs and Minipigs

The peripartum PRL surge begins about two days prepartum and extends through several days postpartum, although it remains significantly greater than those found during most of pregnancy. The prepartum peak of PRL is essential for the onset of lactation, and the decline slowly begins over the initial days postpartum [49]. PRL has indeed been proven to direct growth/differentiation of epithelial cells in the mammary gland throughout gestation, with an experimentally induced late gestational hyperprolactinemia being responsible for higher milk yields [32]. In pigs and minipigs, the hormone relaxin from the corpora lutea has a similar function to placental lactogen, produced in humans and rodents, which is a PRL agonist [42,60]. Regarding the porcine species, most of the studies concerning lactation have been carried out under intensive breeding conditions; there is little information about pigs' lactation in wildlife. Wild sows build a nest for their litters, and the piglets remain in the nests for about two weeks and are weaned after an eight-week lactation. In commercial intensive piggeries, the length of lactation has been truncated to 21–28 days (3–4 weeks), to increase profitability. The important role of milk ejection in lactation is clearly illustrated by the characteristic behavior pattern associated with suckling and the oxytocin release in the domestic sow, resembling what happens in humans. The piglets jostling and nuzzling on the mammary glands induce oxytocin release, followed by rapid ejection of milk from the mammary glands. Piglets have a very short amount of time, in terms of minutes, to obtain all the possible milk from their preferred nipple [49].

3.4. Dogs

Dogs also show a similar physiology of lactation to humans, with the drop in progesterone and estrogen post-parturition, and increased levels of PRL from mid-gestation to weaning, with higher spikes starting at parturition. Placental relaxin secretion starts from mid-gestation, decreases in the third trimester, and drops at parturition [61].

3.5. Mice

Although progesterone signaling controls alveolar proliferation, PRL directly controls epithelial cell differentiation [62]. Its release is essential for the proliferation and functional

differentiation of lobulo–alveolar structures during pregnancy [63]. Moreover, as opposed to humans, PRL has a strong luteotropic action in rodents, promoting progesterone production during pregnancy [44]. The estrogen hormone has receptors in both stromal and epithelial cells, but it is required only in the stroma for proper ductal development. Oxytocin, when released, induces the contraction of the myoepithelial cells surrounding the alveoli and thereby induces milk ejection. Thus, oxytocin is not only necessary for postpartum milk ejection but also for alveolar cell proliferation [62].

3.6. Rats

The physiology of rat lactation is similar to that reported for the mouse. In rats' mammary glands, PRLR expression is low during most of pregnancy and starts increasing on day 21, potentially in response to the pre-partum rise in pituitary PRL release, and continues to increase throughout lactation [64]. In female rats, lactation induces the mobilization of fat stores and a large increase in food intake, depending on the size of the suckling litter. In rats, PRL secretion is suppressed during the second half of pregnancy [65]. The placenta produces the placental lactogen, which binds to PRL receptors and stimulates growth and differentiation of epithelial cells in the glands in the same way as PRL [60].

3.7. Rabbits

Data regarding the physiology of lactation in rabbits are unfortunately relatively poor. Nonetheless, the interest toward this species as a potential model for breast cancer has led to some interesting studies and updated reviews [37]. What is known is that lactation usually lasts up to 4–5 weeks [49], and that the litter only suckles once or twice a day. Additionally, in this species, the key factor for lactation and its maintenance is PRL [50].

4. Colostrum and Milk Composition

Milk and colostrum composition vary greatly among animal species, as reported by a previous review of the literature [66]. Components of milk and colostrum include proteins, lipids, carbohydrates, minerals, vitamins, and cells. The milk components were found to be influenced considerably by the stage of lactation, where these changes differ often from one species to another. In general, colostrum differentiates from milk mainly due to its high immunoglobulins (IgG) concentration. It is important to acknowledge that the different types of placentation and active immunity transport mechanisms, peculiar to each species, highly influence the degree of the colostrum-mediated transfer of immunity. The degree and timing of immunity transfer during pregnancy to the offspring impacts on the importance of colostrum for immunity transfer [24,67]. In humans and NHPs, IgG are transferred to the fetus during the second and the third trimester of pregnancy, while in rodents and rabbits this occurs mainly during organogenesis [67,68]. In other mammalian species, such as dogs and pigs, IgG transfer during pregnancy is minimal; hence, the immunity transfer from the dam to the offspring is essentially lactogenic, with 85–95% of the blood immunoglobulins originating from colostrum transfer [69,70]. Overall, IgG colostrum concentration is specifically high after parturition and rapidly drops. In addition to systemic immune protection, colostrum also plays a major role for the local digestive system due to the presence of IgA, isoenzymes, lactoferrin, white blood cells, and various cytokines. The newborn absorbs colostrum IgG from the digestive tract into the blood stream. The newborns' ability to absorb IgG ends shortly after parturition [51]. Although colostrum has higher concentrations of immunoglobulins in all mammalian species, the concentration of other components may vary between species. Very few papers investigating the milk composition early in lactation were found in our search. In addition, different components and methods were investigated and used at each paper. The main characteristics and differences of the mammary secretion of the species examined for colostrum and "mature" milk are shown in Tables 3 and 4, respectively.

Table 3. Colostrum composition.

Species	Dry Matter %	Protein %	Casein %	Whey Protein %	Fat %	Lactose %	Fe µg/mL	Cu µg/mL	Zn µg/mL	Mn µg/mL	Mg µg/mL	Ca µg/mL	P µg/mL	References
Rats	/	8.6–9.1	/	/	13.6–15.7	2.3–2.6	8.1–9.2	8.6–9.8	13.3–14.2	0.3–0.4	168–180	755–829	/	[71]
Mice	/	/	/	/	/	/	/	/	/	/	/	/	/	/
Rabbits	31.4–33.7	13.5–15.9	/	/	13.7–20.4	1.6–2.1	/	/	/	/	/	/	/	[72]
Dogs	/	12.4–16.2	60.7	39.3	13.2	1.7	3.7	1.3	5	/	128	1363	935	[73]
(mini)Pigs	20.1–26.7	7.7–16.6	1.5–3.4	7.9–14.8	6.4–8	2.8–3.9	/	/	/	/	100	800	1000	[56,74]
NHPs	/	2.2–2.7	/	/	4.3–6.3	7.7–7.9	0.9–2.6	2–4.1	3.5–6.8	/	37.5–61.7	324–347	/	[75]
Humans	11.92	2.6	0.4	1.18	3	5.8	1.1	0.4	4.8	0.01	32	293	159	[76–78]

Data are expressed as ranges or single values; /, data not available.

Table 4. Mature milk composition.

Species	Dry Matter %	Protein %	Casein %	Whey Protein %	Fat %	Lactose %	Fe µg/mL	Cu µg/mL	Zn µg/mL	Mn µg/mL	Mg µg/mL	Ca µg/mL	P µg/mL	References
Rats	27.9–32.8	8.9–9.7	6.4–8	0.9–2.5	14–15.9	1.1–4.1	4–7	1.7–7	9-55	/	158–192	2849–3206	1600–2720	[79,80]
Mice	36.3–39.4	10.1–12.7	/	/	19.3–22.9	2.4–2.8	/	/	/	/	/	/	/	[48]
Rabbits	31.2	10.3	/	/	15.2	1.8	0.003 §	0.002 §	0.02–0.03 §	0.0001 §	0.35–0.45 §	2.71–5.36 §	2.44–3.28 §	[72,81]
Dogs	22.7–26	4.3–9.8	65.8–75.4 *	26.4–34.2 *	2.4–13.4	29.3–40.2	1.8–13.1	0.9–2	4.1–9.6	0.1-0.2	55.8–104.3	1366–2440	914–1401	[52,73,81–83]
(mini)Pigs	18.8–22.7	5–7.5	2.7–3.6	2.4–5.4	7–10.1	4.3–5.6	/	/	/	/	105	2000	1420	[56,74]
NHPs	12.2–14	1.3–2.3	45 *	55 *	3.3–6.2	4.8–9.1	/	/	/	/	34	380	152	[81,84–87]
Humans	12.6	1.2	0.3	0.7	4.1	7	0.5–1.8	0.2–5.2	0.7–3.8	0.01-0.03	25–33	230–310	130–190	[88–90]

Data are expressed as ranges or single values; /, data not available; *, % on total proteins; §, these data are expressed in g/kg.

4.1. Humans

As for the other mammals, the composition of breast milk is affected by different factors and, depending on the individual, changes over the course of a single breastfeeding session, of a day, and through lactation [77,89]. One of the key factors seems to be represented by the maternal diet: indeed, dietary intake of different nutrients, particularly fatty acids and some micronutrients, is related to their content in breast milk composition, but does not affect macro-nutrient composition [91]. Generally speaking, fat concentrations tend to increase not only over the course of a day, but also during the same breastfeeding session [89]. On the other hand, the overall protein and amino acid contents show a marked decreasing trend over time during the first year of lactation [77]. One of the few components that seems to remain relatively stable for the entire duration of lactation, except for colostrum, is lactose [77]. Regarding minerals, magnesium, phosphorus, and calcium show significant lactation-stage-specific differences and high inter-individual variations, while manganese, copper, and iron remain relatively constant during all lactation stages. Zinc concentrations, overall, show a decreasing trend as lactation progresses [77].

4.2. NHPs

Non-human primate milk is relatively diluted: it generally consists of <15% dry matter, with about 7% sugar, ≅3–6% fat, and ≅1–2% proteins, and changes over the course of lactation. It is important to acknowledge that, amongst different NHP species, great differences in milk composition can be observed, mainly imputable to the length of lactation, frequency of feeding, and milk yield [92,93]. Most studies investigating the NHP milk composition used the rhesus macaque, while few analyses of the milk composition of cynomolgus monkeys, which is the most common NHP in research, were found. In rhesus macaques (*Macaca mulatta*), both fat and protein contents increase as infants age, in the light of the higher demand in energy. Such increase in energy content seems to be related to lower milk yields. Out of the different components, fat percentages show the highest inter-individual variation within the same species, while lactose levels are relatively stable [86]. However, differences in milk composition among prosimians may be related to differences in maternal care: species that carry their offspring produce more dilute milk, with higher yields, when compared to species that usually leave newborns for prolonged period. Lorises, bushbabies, and potentially cheirogaleids produce relatively rich, energy-dense milks in comparison with anthropoid primates, such as rhesus macaques (*Macaca mulatta*), white handed gibbons (*Hylobates lar*) and gorillas (*Gorilla gorilla gorilla*) [92].

4.3. Pigs and Minipigs

When compared to mature milk, colostrum has higher concentrations of protein, particularly immunoglobulins, some minerals (particularly copper, iron, iodine, and zinc) and vitamins, hormones, and growth factors. Lactose is present in lower concentrations in colostrum than in mature milk. Milk fat concentration transiently increases during the period from day 2 to day 4. The composition of milk after approximately day 7 to day 10 is relatively stable for the remainder of lactation. As for other species, maternal diet can affect some milk components, including concentrations of fat, fat-soluble vitamins, and some minerals, as well as proportions of specific fatty acids. Some components of sow milk also are affected by genetics, parity, colostrum and milk yield, and ambient temperature [56].

4.4. Dogs

Early studies showed that dog milk composition might change with breed. For this summary, we focused on the beagle dog because it is the commonly used breed for research purposes. To summarize, the concentration of iron, zinc, calcium, protein, and fat showed patterns that were influenced by the stage of lactation. The concentration of copper, manganese, magnesium, and carbohydrates were not significantly affected. Adkins et al. [73] found that protein concentration was high in colostrum, decreasing significantly by day 21, and then slightly increased throughout the duration of lactation.

This pattern of decreasing protein concentration during lactation is similar to humans. However, Lönnerdal et al. [82] reported that protein concentration increased over time. As for humans, a decreasing pattern in the concentration of all amino acids with the increasing lactation stage was observed. The lipid content does not show remarkable changes during the lactation period. Slight non-significant decreases were observed between days 14 to 28. The lipid concentration in dog milk was higher than that reported in humans [73]. Lactose levels were low in colostrum, increasing gradually until day 28, followed by a slight decrease. Iron concentration increased significantly from day 1 to day 3, and then gradually decreased by day 42. This is in contrast to humans, where iron concentration is high in the colostrum and then gradually decrease during lactation [73]. Lönnerdal et al. [82] reported that the zinc concentration decreased throughout lactation, while Adkins et al. [73] reported that zinc concentration slowly increased from day 1 to day 14, and then decreased by day 42. Zinc levels were higher than those reported for humans [73,82]. Copper concentration was slightly higher in early lactation and then gradually decreased throughout lactation or remained unchanged. Copper levels were generally higher than those reported in humans. Milk calcium concentration was lower in colostrum but increased thereafter, peaking on day 35. Calcium levels were significantly higher than those reported for humans [73,82]. Magnesium concentration was highest in the colostrum but rapidly decreased by day 3 and remained relatively constant during lactation. Concentration of phosphorus showed a very mild increase from day 3 to day 28 [73]. The iron concentration in the dog milk is influenced by the stage of lactation, with values decreasing over time. This is similar to what was reported in other species; however, the iron concentration in dog milk was found to be considerably higher than that of human milk. The manganese concentration was not found to be influenced by the stage of lactation, and its levels were higher than reported for human milk. The fat content of canine milk was found to be influenced by the stage of lactation, with concentrations increasing during the first part and decreasing during the last part [82]. The level of carbohydrates was fairly constant and did not show a strong developmental pattern.

4.5. Mice

Very few papers have been published on mouse milk composition. The composition of mouse milk can vary considerably between mouse strains. In general, the analysis on mouse milk is challenging due to the small sample volume and the high fat content. Hopefully, the improvements of new analytical methodologies should fill in our knowledge gaps, as suggested by the work of Stewart and Davis [28]. Crude protein levels did not change during lactation [48,94,95]. Crude fat increased from day 3 of lactation to day 14 and remain stable until day 18 [48,94], while in another study the fat content decreased from early to mid-lactation [96]. Lactose content increased with lactation day [48,95]. Great variability was noted in lactose content and crude protein between strains of mice.

4.6. Rats

There are characteristic differences in the nutrient content of milk among strains of rats, particularly for changes in the lactose and fat content of the milk during the lactation period. In general, rat milk elements (iron, copper, zinc, manganese) show a similar pattern of high initial levels, mainly during the colostrum phase, that decreases throughout lactation. The concentration of some elements increases during the last phase of lactation (iron, manganese). The iron concentration of rat milk, considerably higher than that found in human milk and much greater than its plasma levels, rapidly decreases during the first part of lactation (approx. 40% drop). Thereafter, it continues to decrease but in a less pronounced manner and increases in the last days of lactation (days 25–28). The decreasing pattern was also found in humans, although the percentage decrease is not that high. Colostrum copper concentration is considerably higher than that of humans and decreases until day 11; it then remains relatively unchanged until the end of lactation. The change in copper concentration pattern is similar to that observed in humans, however

the copper concentration in rats' milk is much higher. The same applies to zinc, showing a similar pattern to humans despite higher overall levels. The concentration of manganese decreases significantly from day 0 to day 12 and remains low, but increases to nearly initial levels by the latter days of lactation. A similar pattern of manganese concentration was reported in humans. In contrast to other elements, the concentration of manganese in rat milk is not considerably higher than that of humans. Magnesium concentration was fairly stable during early and mid-lactation and decreases during late lactation. The protein and calcium concentration increase steadily during lactation until day 24 and decreases at the end of the lactation period. The similar patterns of calcium and protein is likely due to the fact that the major protein of rat milk is casein, which is well known for its calcium binding capability [97]. In contrast to rats, human calcium levels decline during lactation. Carbohydrate concentration increases during the first half of lactation, then decreases during the second half. In humans' milk, the carbohydrate level is much higher than in rats. In humans an increase in milk carbohydrate is also found in the early lactation period, but there is no decrease at later stages of lactation. While lactose is by far the major carbohydrate in human milk, rat milk may contain significant amounts of neuraminlactose. Quantitatively, the most important constituent in rat milk is fat. Similar to humans, the fat content of the rat milk did not exhibit a strong pattern during the lactation period [71].

4.7. Rabbits

Kits are weaned at the age of 4–5 weeks and are exclusively dependent on milk until lactation day 18–19. Rabbit milk yield corresponds to kits' weaning stage and reaches its peak around lactation days 17–21. It is important to remember that rabbits only nurse kits once or twice a day, strongly impacting the nutritional value of the milk. In general, rabbits' milk is concentrated with fat, protein, and energy, but nearly absent of lactose, and the composition does not vary significantly between most breeds [72]. As in other mammalian species, the colostrum has higher protein content due to high immunoglobulin levels. This increases the dry matter value of colostrum relative to rabbit milk. Protein content decreases along with the increase in milk yield during lactation. Apart from protein content, the composition of rabbit milk is quite stable during the second and third weeks of lactation. The changes in composition in the later stage of lactation are closely related with the decrease in milk yield. Mineral element composition changes substantially after the lactation peak. In general, rabbit milk is rich with calcium, sodium, and potassium. Calcium concentration, and to a lesser extent phosphorus, increases with the progressing lactation stage, while the effect on potassium and sodium is less clear and there are different data values reported in different papers. Magnesium content increases with lactation stage, while zinc, copper, iron, and manganese decrease gradually in concentration as lactation progresses [72].

5. Discussion

5.1. Anatomy of the Mammary Gland

As expected, from a gross anatomy point of view, the variations between the analyzed species are very high. When looking at the number and the position of glands, NHPs better resemble the human situation, with differences only in the ducts. The number of teats is directly related to the number of the offspring [98]; therefore, such a situation was to be expected. Pigs are on the opposite side of the spectrum because, depending on the breed and in light of the pork production requests, sows can deliver up to 18 piglets. The human mammary gland, out of those taken into account, is the one with the highest number of canal/ducts, followed by the canine species. A peculiar situation can be noted in rodents, where only one canal per teat is present. When selecting a relevant animal model for trials involving lactation, the similarity in gross anatomy is not necessarily one of the key decisional factors. Indeed, having a higher number of mammary glands and teats often allows for easier sampling procedures and, potentially, higher volume specimens.

5.2. Physiology of Lactation

No major discrepancies were noted regarding the physiology of lactation amongst the analyzed animals. Indeed, hormonal inputs and pathways are pretty much conserved, with PRL and oxytocin being pivotal. Nonetheless, PRL seems to play different roles in rodents when compared to the other species analyzed in the present literature review. When looking at the most important veterinary textbooks for such topics, the bovine species is always the most included and analyzed within the physiology of lactation chapters, because its role in the milk industry for human consumption is undeniable. This represents a relative limitation for experimental animals, and in particular laboratory animals, because the peculiar gastrointestinal apparatus of ruminants changes the metabolic scenario of milk production. Several knowledge gaps need to be filled regarding the physiology of lactation, both at systemic and molecular levels, including in-depth characterization of hormonal mechanisms of action and environmental influences, among others.

5.3. Colostrum and Milk Composition

The comparison of colostrum/milk compositions between the different species can be hard to interpret in light of several technical and physiological issues. Indeed, despite representing the basis of today's knowledge, a lot of the retrieved papers are relatively old, often relying on low sample sizes and less accurate analytical tools when compared to modern analyses. In addition, different components and methods were investigated and used in each paper. Moreover, high inter-individual variations, mainly qualitative, within the same species are extremely common, and depend on a wide variety of factors including maternal nutritional status and diet, number of newborns, or duration of lactation. Colostrum of all species contains high levels of IgG and other immune factors which increase the protein content. The level of IgG decreases within a few days post-partum. As for the other components of both milk and colostrum, they vary between species and the NHPs seem to better resemble humans' mammary gland secretions, which are definitely more diluted when compared to other species such as rabbits, rats and mice. This finding was to be expected because, as already mentioned, the number of newborns and maternal care are amongst the key factors. Nonetheless, (mini)pigs show a relatively close similarity with humans' milk gross composition, despite their capability to produce high litter sizes.

5.4. Practical Considerations

Choosing an animal model for a particular trial is always difficult and often implies the necessity to cope with knowledge and information gaps. The topics covered here represent the starting point for a conscious decision, because anatomical and physiological similarities to humans are the basis of a high translational experimentation value. Looking at the results, NHPs, as expected, seem to represent the best model to enroll in studies regarding lactation. Nonetheless, other factors have to be considered. In the last decade, the scientific community has come to the agreement of using experimental NHPs only when strictly necessary [99]. Moreover, trials involving NHPs are expensive, long, and due to ethical considerations have low sample sizes that can undermine the outcome data. Finally, collecting milk samples from such species could be extremely difficult in light of the maternal behavior. Ease of sample collection should always be an important factor when designing animal trials, because choosing a "difficult" species may lead to failure, especially in "longer" trials that require un-sedated animals and/or repeated samples. Smaller animals such as rodents and rabbit are not necessarily the best choice for lactation studies. Indeed, in order to collect milk from rodents, one of the few options is the euthanasia of pups to collect gastric content right after suckling. Other methods, such as using mini-pumps to collect milk directly from the mother, often require pharmacological support to increase milk volume, a strong bias in drug lactation transfer studies, and still leads to small volume of samples. Furthermore, when compared to humans, their milk composition was quite different and discrepancies in PRL production and functions were highlighted. In such a scenario, larger animals such as dogs and pigs seem to be a good fit for lactation

trials. With regard to dogs, it is important to acknowledge that, despite their relevance for regulatory toxicology studies, their use for biomedical research often raises strong criticism and ethical issues in public opinion, especially in Europe. On the other hand, the enrolment of pigs in research trials seems to be more widely accepted and is considered as a valid alternative when anatomical/physiological differences are not relevant [100]. Minipigs, in particular, offer all the advantages of conventional pigs including genetic and metabolic similarities to humans, avoiding the main problem represented by the size. Their use in the biomedical setting is well established and recognized, and the availability of in-depth physiological characterization and the related physiology-based models, vital for results interpretation, is increasing. Moreover, Göttingen Minipigs are specifically produced for biomedical purposes, with a high standardized genetic background and health status.

6. Conclusions

In conclusion, the present analysis of the literature confirmed the complexity of the decisional process behind the choice of an animal model for in vivo trials. For some of the reviewed species, data were either poor or completely missing, highlighting the necessity to generate more physiological background studies for species that are routinely used in laboratory settings. Overall, pigs, and in particular minipigs, seem to represent the better choice when looking at both physiological similarities with humans and the feasibility of lactation trials.

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2. First step

ConcePTION Work Package 3 (WP3) is assigned to the development of a preclinical testing platform to determine pharmaceuticals transfer into human breast milk and consequent neonatal exposure. To comply with this purpose, multiple strategies have been undertaken. Amid WP3, the Task 3 is deputy to establish a trustworthy animal model to enroll in *in vivo* lactation trials translatable to humans. An accurately aimed literature review of mammalian species mainly used in biomedical research, highlighted pigs as suitable to setting up the *in vivo* lactation model (see **1.Preliminary step**). In parallel, another WP3 Task was appointed to the validation of a reliable isolation method for porcine Mammary Epithelial Cells (pMECs) to evaluate the swine species as a strong translational model of the mammary epithelial barrier.

The veterinarians research team of the Department of Veterinary Medical Sciences, Alma Mater Studiorum – University of Bologna, performed the first in vivo trial within ConcePTION project. Overall, 6 lactating sows were used, 50% conventional hybrids and 50% Göttingen minipigs, to get a very first hunch on the feasibility of this innovative translational model. Amoxicillin was selected as opening molecule because a moderate amount of pharmacokinetic (PK) and pharmacodynamic (PD) data in swine were already available.

This introductory trial led the foundations for an innovative porcine model for translational lactation studies, opening the pathway to include such *in vivo* and *in vitro* model in a pipeline of PK testing prior drug approval.

- Deliverable 3.3 "*Report on characterization in vitro human or animal mammary epithelial cell cultures models, including comparison between in vitro models*"

Ref. Ares(2021)5133907 - 16/08/2021

IMI2 821520 - ConcePTION

ConcePTION

WP3 – Determination of drug transfer and infant drug exposure during lactation: generation of quantitative and translatable data

D3.3 Report on characterization in vitro human/animal mammary epithelial cell cultures models, including comparison between in vitro models

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- Deliverable 3.5 "*In vivo data on lactation transfer in one or more animal species*"

IMI2 821520 - ConcePTION

ConcePTION

WP3 – Determination of drug transfer and infant drug exposure during lactation: generation of quantitative and translatable data

D3.5 In vivo data on lactation transfer in one or more animal species

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IMI2 821520 - ConcePTION

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WP3 – Determination of drug transfer and infant drug exposure during lactation: generation of quantitative and translatable data

D3.5 In vivo data on lactation transfer in one or more animal species

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Document History

Version	Date	Description
V1.0	29 Mar 2022	Draft
V2.0	11 Apr 2022	Final Version

Abbreviations

EDTA	Ethylenediamine tetraacetic acid
Ga	Gauge
ID	Identification
IM	Intramuscular
IV	Intravenous
LLOQ	Lower limit of quantification
NSAID	Non-steroidal anti-inflammatory drugs
PBPK	Physiologically based pharmacokinetic
PD	Pharmacodynamics
PK	Pharmacokinetic
SEM	Standard error of the mean
SID	Semel in Die (Latin: Once A Day)
ULOQ	Upper limit of quantification
WP	Work package

Content

Abstract

The aim of task 3.3 of the ConcePTION project is to develop and characterise a relevant i*n vivo* model for drug passage from maternal blood to human breast milk. The chosen species to perform such non-clinical trial was the porcine one, as reported in the Deliverable 3.2 of the same project. In the initial trial, amoxicillin was chosen as the first molecule for a variety of reasons including alignment with other Work Packages (WPs) performing studies on human breastmilk. Additionally, since amoxicillin is commonly used in pigs, the choice of the route of administration, the administration interval and the doses were supported by reliable background data. Six sows were used for this amoxicillin trial: 3 conventional commercial hybrids and 3 Göttingen Minipigs. The trial started at the beginning of the second week of lactation and lasted up to piglets weaning (28 days after birth). Sows were administered IM with 7mg/kg of amoxicillin (SID) for the entire duration of the trial; on the first day a PK analysis was performed to check the consistency with literature data. For the rest of the trial, sampling days were divided into “sow days”, consisting of 4 matched blood/milk samples, and “sow+piglets days”, consisting of 2 matched milk/blood samples from the sow plus blood from 4 piglets (2 piglets sampled before amoxicillin and 2 piglets sampled 30 minutes after sows’ sampling). To quantify amoxicillin, samples were frozen, shipped on dry ice and analysed by BioNotus. Amoxicillin was always quantifiable in sows’ samples: the highest plasmatic concentrations were recorded 2h after dosing, while the highest milk concentrations 4h after. Out of the 136 blood samples collected from piglets, only 9 showed amoxicillin levels above the detection limits. The latter finding, along with the recorded milk peak at 4 hours after administration, seems to suggest the need for additional sampling time points for piglets. Overall, the study design used for this initial amoxicillin trial has allowed to highlight both strengths and critical points of this new *in vivo* platform to test infant drug exposure through milk. The procedures were feasible and well tolerated by animals that gained confidence and trust during the preliminary training sessions. This is extremely important when assessing the overall ethical impact of the trials.

Methods

1.1 Animals

The preliminary trial for Amoxicillin was performed at the University of Bologna on a total of 6 sows: 3 conventional hybrids and 3 Göttingen Minipigs. As previously stated, conventional pigs were included for several reasons, including the experience of the research team with such breeds, the usually higher litter size and the volume of collectable samples. Conventional pregnant sows were purchased from a local farm, SUIMAX di Massimo Ferri (Via San Michele 718, Valsamoggia 40056 BO, Italy), chosen based on the microbiological status of the facility and for the reproductive track records. Göttingen Minipigs pregnant sows were provided by Ellegaard Göttingen Minipigs (Soroe Landevej 302 4261 Dalmose, Denmark), which contributed as breeding facility within the project framework. All piglets included in the study were born from the above-mentioned sows in the experimental facility of the ANFI-ASA Unit, Department of Veterinary Medical Sciences, Alma Mater Studiorum – University of Bologna (via Tolara di Sopra 50, Ozzano dell'Emilia 40064 BO, Italy).

Sows were transferred to the experimental facility 1 month prior the expected delivery date (calculated based on the insemination/mating date and ultrasound pregnancy scan) and moved to the farrowing pen one week before. Animals were checked at least twice a day for health status. Farrowing was not pharmacologically induced, as this often leads to complications, but remotely assisted by trained veterinarians.

Animals were fed a standard diet according to the breed: Basic Micropigs (9AB17) by Mucedola s.r.l. (Settimo Milanese 20019 MI, Italy), which is specialized in dedicated formulas for lab animals exclusively used for experimental purposes; while commercial sows received a formula specifically made for breeding animals, produced by a local vendor (Molini Popolari Riuniti, Ellera-Umbertide 6019 PG, Italy). Both feeds were in the form of pellets, since its production process is suitable for preserving the organoleptic and hygienic characteristics of the product for a long time (24 months from the date of production). Drinking water was provided ad libitum, while the daily feed ratio was divided into two portions, early morning (7:30 am) and afternoon (3:30 pm); feed total amount was adjusted during the course of lactation, to cope with the considerable metabolic energy expenditure of the sows. Light/dark cycle was set at 12/12h with a min Lux value during light hours of 40. Temperature was set at 21 ±1°C to meet sows thermal needs. With regards to piglet, two heat lamps were placed in dedicated areas of the farrowing crate to reach 32 ±1°C.

Piglets were individually identified with ear tags, and iron dextran (100mg) was administered IM within the first 72h of life.

1.2 Training

A key factor for the success of *in vivo* trials is animals' behavior, that can be shaped through the consistent use of positive reinforcement training, also fulfilling the 3Rs principle for animal welfare.

Clicker training, first described for dogs, is accepted worldwide as one of the most effective methods for teaching basic obedience to different animal species and to direct their intelligence toward productive and positive activities. It has been adapted to numerous other species, including pigs. The method is based on positive reinforcement, precisely with the association of the double-click sound produced by a specific device, with a reward, such as a tasty snack or a toy. One of the biggest challenges in pig training, especially when looking at conventional animals, is getting the animals to trust the operator and understand that no harm is involved. This is also why, alongside microbiological evaluations, it is important to select a supplier with extremely high welfare standards. Once they understand the training session entails no distress, it becomes a welfare enrichment itself. When transferred to the experimental facility, pregnant sows (both conventional and Minipigs) were gradually accustomed to the trainers, first by just introducing a human presence in the room with food rewards; these first acclimatization steps were short (just few minutes) but were done daily, to build trust and a bonding relation. As a positive reinforcement, animals were given a food reward (apple slices, apple juice and yogurt were always very welcome). After the first week, trainers started to approach the animals: pregnant sows were gradually accustomed to follow the trainers and being manipulated especially at the level of mammary glands and ears. Training sessions were short (20 min) but consistent and repeated daily (Monday-Friday). After some repetitions, the action-click-reward association became a conditioned stimulus that could keep the animals motivated to accomplish different tasks while having fun in the meantime. As demonstrated by previous studies, swine positive emotions could be indicated by play, barks and tail movements, while negative behavior is suggested by freezing, defecating/urinating, escape attempts, high-pitched vocalizations and ear movements. During the training sessions all the animals showed well-being associated activities, and, after the routine was established, they were easily manipulated and seemed to enjoy the training session, an additional occasion to get out of the cage and explore the environment.

1.3 Long-term catheter insertion

Towards the end of the first lactation week, conventional and Minipig sows were deeply sedated by an intramuscular injection (Tiletamine/Zolazepam 3.5-5 mg/kg; Zoletil 50/50 Virbac srl) for the peripheral insertion of a long-term catheter through an auricular vein. Oxygen was provided during the procedure through a breathing mask. During the entire duration of the procedure, which always lasted less than 60 min, support fluid therapy (Ringer Lactate 10 ml/kg/h) and the usual anaesthesia monitoring were provided (ETCO2, SpO2, Temperature, ECG, NIBP).

Catheterizations were performed following standard surgical sterility guidelines: clipped, neat, disinfected area dedicated, sterile surgical draping, gloves and instruments (packed and autoclaved for each animal); surgical field prep consisted of 3 alternating scrubs of a chlorhexidine scrubbing solution and 70% alcohol. Ear veins (*v. auricularis caudalis* or *v. auricularis intermedia*) are suitable for this kind of IV long term access, despite course, branching and size can differ dramatically from

animal to animal. Seldinger's over-the-wire technique was employed for peripheral auricular vein catheterization: the vessel was punctured with a dedicated sharp hollow needle which was then removed with the plastic sheath left in place to allow the guidewire to be advanced through it for at least 30mm. Once the wire was inserted without major resistance, the sheath was removed and replaced with a vessel dilator. Finally, upon dilator removal, the catheter was slid over the wire and into the vein, sutured to the skin and further fixed by means of an IV securement dressing integrated with chlorhexidine gluconate gel pad (Tegaderm CHG, 3M). Heparinized saline (300IU/ml) was used as lock solution to ensure patency on a daily bases and after every blood collection. Catheter sets were purchased by Mila International: MILACATH Guidewire (SA1925) - Single Lumen - 19Ga (3.5Fr) x 25cm (10in) for Göttingen Minipigs sows; MILACATH Guidewire (SA1420) - Single Lumen - 14Ga (6Fr) x 20cm (8in) with integrated extension set, for commercial breed sows.

1.4 Study design and amoxicillin dosing

On the first day of the trial, sows were administered IM with 7mg/kg of amoxicillin (Clamoxyl® RTU; Pfizer) and a PK analysis was performed. A total of 11 blood samples were collected:

- Before amoxicillin administration
- 30min after administration
- 60min after administration
- 90min after administration
- 2h after administration
- 3h after administration
- 4h after administration
- 5h after administration
- 6h after administration
- 12h after administration
- 24h after administration

For the remaining 3 weeks of lactation, sows were always administered IM 7 mg/kg of amoxicillin (SID); injection were performed at the common preferred area for adult pigs, just behind the base of the ear, alternating the side every day. Animals were distracted and rewarded with food during injection, which was quick. Sows' weights were recorded at least once a week to adjust amoxicillin dosage.

The trial was structured as follows:

- SOW DAY: 4 matched milk/blood samples (before amoxicillin, 2, 4 and 8h after); twice a week

- SOW+PIGLET DAY: 2 matched milk/blood samples (before amoxicillin and 2h after) from the sow plus blood from 4 piglets (2 before amoxicillin and 2 piglets 30 minutes after sows' sampling); twice a week.

	I19			I20			I21		
date	post partum	lactation week	procedure	post partum	lactation week	procedure	post partum	lactation week	procedure
13/04/2021							farrowing		
14/04/2021	farrowing						P1		
15/04/2021	P1			farrowing			P2		
16/04/2021	P2			P1			P3		
17/04/2021	P3			P2			P4		
18/04/2021	P4			P3			P5		
19/04/2021	P5		Cat	P4		Cat	P6		Cat
20/04/2021	P6			P5			P7		
21/04/2021	P7	amoxi	PK	P6	amoxi	PK	P8	amoxi	PK
22/04/2021	P8	amoxi	Sow	P7	amoxi		P9	amoxi	Sow
23/04/2021	P9	amoxi	Sow+Piglets	P8	amoxi	Sow	P10	amoxi	Sow+Piglets
24/04/2021	P10	amoxi		P9	amoxi		P11	amoxi	
25/04/2021	P11	amoxi		P10	amoxi		P12	amoxi	
26/04/2021	P12	amoxi	Sow+Piglets	P11	amoxi	Sow	P13	amoxi	Sow+Piglets
27/04/2021	P13	amoxi	Sow	P12	amoxi	Sow+Piglets	P14	amoxi	Sow
28/04/2021	P14	amoxi		P13	amoxi		P15	amoxi	
29/04/2021	P15	amoxi	Sow	P14	amoxi	Sow+Piglets	P16	amoxi	Sow
30/04/2021	P16	amoxi	Sow+Piglets	P15	amoxi	Sow	P17	amoxi	Sow+Piglets
01/05/2021	P17	amoxi		P16	amoxi		P18	amoxi	
02/05/2021	P18	amoxi		P17	amoxi		P19	amoxi	
03/05/2021	P19	amoxi	Sow+Piglets	P18	amoxi	Sow	P20	amoxi	Sow+Piglets
04/05/2021	P20	amoxi	Sow	P19	amoxi	Sow+Piglets	P21	amoxi	Sow
05/05/2021	P21	amoxi		P20	amoxi		P22	amoxi	
06/05/2021	P22	amoxi	Sow	P21	amoxi	Sow+Piglets	P23	amoxi	Sow
07/05/2021	P23	amoxi	Sow+Piglets	P22	amoxi	Sow	P24	amoxi	Sow+Piglets
08/05/2021	P24	amoxi		P23	amoxi		P25	amoxi	
09/05/2021	P25	amoxi		P24	amoxi		P26	amoxi	
10/05/2021	P26	amoxi	Sow+Piglets	P25	amoxi	Sow	P27	amoxi	Sow+Piglets
11/05/2021	P27	amoxi	Sow	P26	amoxi	Sow+Piglets	P28	amoxi	Sow
12/05/2021	P28	amoxi		P27	amoxi				
13/05/2021				P28	amoxi	Sow+Piglets			

Figure A. Trial calendar for the Göttingen Minipigs sows.

1.5 Samplings

Blood samplings from sows were carried out relatively easily due to the previously described training: animals, distracted by the food reward and accustomed to being touched on the ears, did not care about the operator collecting blood from the catheter. The latter allowed for a pivotal refinement of the study as no pain was involved in this procedure. Yet it has to be acknowledged how commercial sows occasionally showed mild aggressive behavior aimed at protecting the litter; this issue was never encountered with Göttingen Minipigs sows. Blood was collected into EDTA tubes, upon elimination of the residual lock solution within the catheter lumen together with the first ml of blood to avoid contamination. Right afterwards, the catheter was flushed with saline solution and locked again with heparinized saline. The procedure was always performed by two operators, one in charge of providing the animal with the reward (usually apple juice) and the second working on the catheter. Sample were immediately centrifuged (15min, 1800rcf, 4°C) to obtain plasma, which was divided into 500µl aliquots and stored at -80°C.

To avoid excessive stress in piglets, blood was collected from the femoral artery upon light sedation achieved by means of Sevoflurane administered through a breathing mask in 100% O2. In order to allow for the potential amoxicillin present in milk to be absorbed into piglets' bloodstream, the samplings were actually performed 30 min after maternal samplings (that always coincided with

nursing).

As for milk samples, the procedure was much more challenging, since the ejection period is very short in pigs and happens only upon intense stimulation of the udder by the litter. Considering the operators would have to wait for the piglets jostling and nuzzling on the mammary glands to induce oxytocin release, pivotal for milk ejection, the research team decided to administer sows with exogenous oxytocin (10-20 IU, IM). This allowed for more precise samplings in terms of timing as matched blood/milk collection was required for this preliminary study. Milking was performed manually, as fast as possible, and directly along the length of the sow's nipple; indeed, massaging the udder does not allow for a good sampling and, as of today, no commercial mechanical devices are available for the chosen species. During the collection of the milk samples, piglets were not separated from the sow, therefore the operators had to compete with the litter to access a teat. Fortunately, piglets tend to establish a preference for a given nipple during the first lactation days, and only aim at that one when nursing. This allowed the operators to know, for each sow, where to collect from. Milk samples were immediately placed on ice, aliquoted as for plasma and stored at -80°C. At the end of the 6 trials, samples were shipped on dry ice to Bionotus for amoxicillin quantification.

Results

During the trial, it was always possible to easily access the ear catheter and the mammary glands for blood and milk collection, respectively. Despite the vulnerable post-partum period, sows have always maintained cooperative behavior towards the trainers, even when piglets were momentaneously removed to perform the blood sampling.

Unfortunately, during the first day of dosing and PK samplings, one of the Göttingen Minipigs sows (ID: I20) became severely lethargic and hyperthermic. The animal was immediately treated with NSAIDs (Flunixin Meglumine, 2.2mg/kg) and IV fluid therapy (Lactated Ringer, 5 ml/kg/h) and strictly monitored; metoclopramide (0.3mg/kg IM) was administered since gastric motility seemed stopped, with negative auscultation, anorexia and failure to defecate. Ellegards' veterinarians were consulted to confirm the correct therapeutic approach. X-ray and ultrasound scans revealed a sever gastric dilation potentially due to overfeeding, thus a gastric washing under general anesthesia (Proposure® 4mg/Kg) was performed. The sow fully recovered within 24 hours and never stopped nursing. However, to avoid potential biases to the trial and results, the sow only re-entered the trial one week after (P15, at the third lactation week). Nonetheless, amoxicillin administration was never interrupted so that the samplings would resemble the ones performed in the other animals.

The results of the bioanalytical assays performed to quantify amoxicillin in both plasma and milk along with the analyses validation and methodology can be found in Bionotus report from Armoudjian Y.

and Lin Q. (Appendix 1; Determination of Amoxicillin in Pig Plasma and Milk using LC-MS/MS; 2021). The lower limit of quantification (LLOQ) was 10 ng/mL, the upper (ULOQ) 10000 ng/mL.

The following tables and graphs represent the averaged data of both conventional and Göttingen Minipigs sows, as no significant differences were recorded between breeds. Generally speaking, amoxicillin was always above the quantification limits in sows' plasma and milk, with the expected exception of the first plasma sample (collected before the first amoxicillin dosing). This finding confirms that amoxicillin is consistently able to cross the blood/mammary barrier into milk.

The PK analyses performed on sows on the first dosing day are represented in **Figure B** (data from I20 were excluded due to the complications during the PK day, see above). At Tmax, the mean plasmatic amoxicillin concentration was 1.44 µg/ml. Amoxicillin was also quantifiable in plasma 24h after dosing, although in very low concentrations (mean 0.097 µg/ml).

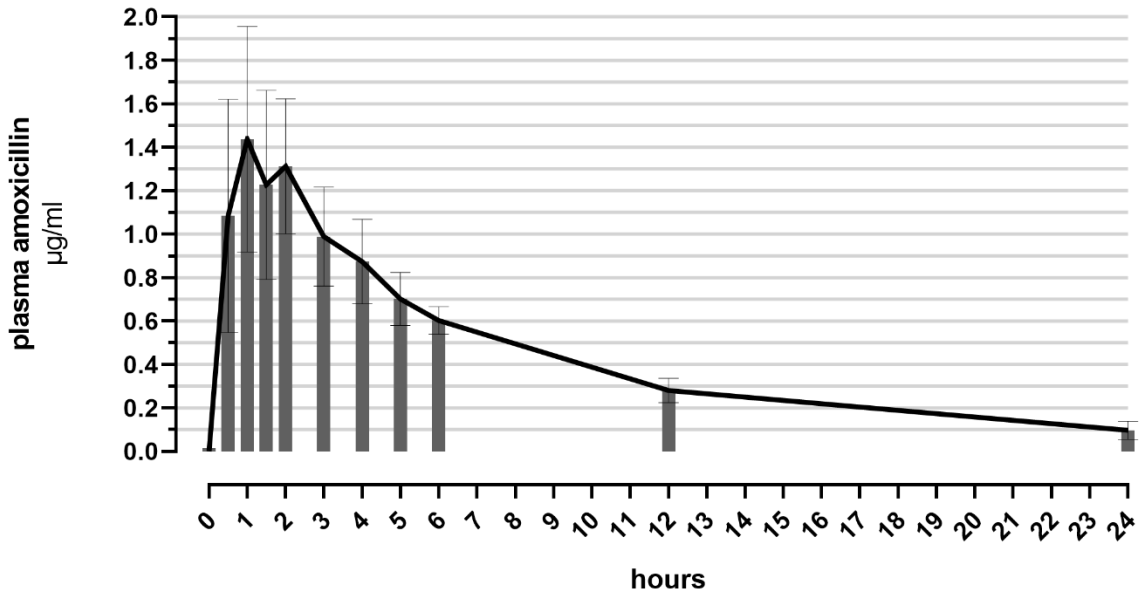

Figure B. Plasma PK of amoxicillin (7mg/kg IM, SID) in conventional and Göttingen Minipigs sows on the first day of dosing. Histograms represent the mean; error bars represent the SEM (standard error mean).

Descriptive statistics for the quantification of amoxicillin into maternal plasma and milk samples are reported in **Table 1** and **2** respectively.

Table 1. Amoxicillin quantification (µg/mL) in maternal plasma samples.

	n	Min µg/mL	Max µg/mL	Mean µg/mL	SEM µg/mL
Before administration	65	0,014	1,849	0,295	0,034
2h after administration	65	0,274	4,977	1,351	0,090
4h after administration	34	0,259	2,349	0,998	0,074
8h after administration	32	0,218	1,726	0,785	0,058

n indicates the number of samples

Table 2. Amoxicillin quantification (µg/mL) in maternal milk samples.

	n	Min µg/mL	Max µg/mL	Mean µg/mL	SEM µg/mL
Before administration	56	0.012	2.039	0.125	0.037
2h after administration	65	0.014	1.607	0.217	0.037
4h after administration	32	0.010	2.583	0.290	0.083
8h after administration	31	0.026	0.390	0.153	0.019

n indicates the number of samples

When looking at amoxicillin concentrations in sows matched blood/milk samples collected before administration, 2, 4 and 8h after, the trends differ between the matrices (**Figure C**). Indeed, the highest plasma concentrations were recorded 2h after IM amoxicillin administration, while the highest milk concentrations were 4h after. For plasma, all experimental time points statistically differed between each other (Kruskal-Wallis test, Conover post-hoc test; $p<0.0001$); as for milk, all the time points after administration statistically differed from the pre-administration one (Kruskal-Wallis test, Conover post-hoc test; $p<0.0001$), but not between each other. The results of the statistical evaluations are indicated by different letters in **Figure C**.

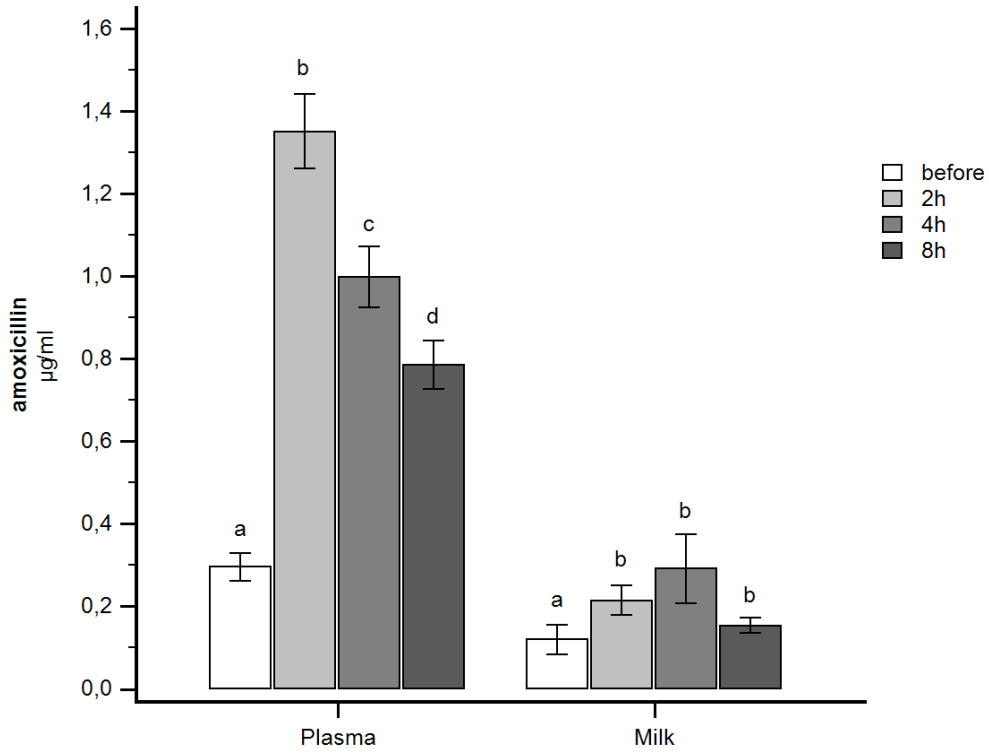

Figure C. Amoxicillin concentrations (µg/mL) in sows' plasma and milk samples at the four experimental time points. Different letters indicate statistically relevant differences ($p<0.05$). Histograms represent the mean; error bars represent the SEM (standard error mean).

Out of the 136 blood samples collected from piglets, only 9 showed amoxicillin levels above the quantification limits (LLOQ 10 ng/ml, ULOQ 10000 ng/ml): five were collected before maternal dosing and four 2h afterwards (**Figure D**). Thus, no inferential statistical evaluation was performed.

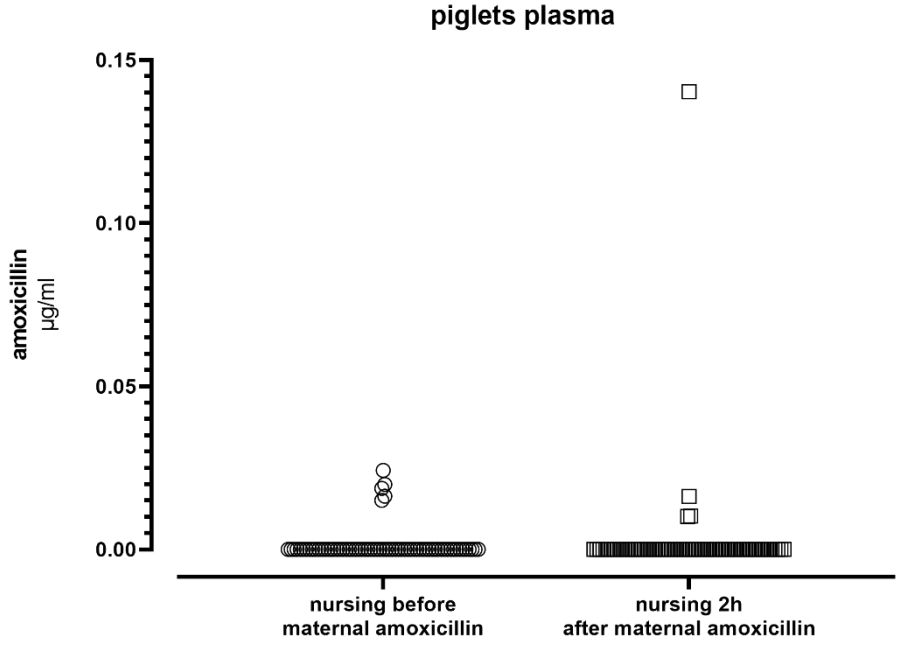

Figure D. Scatter plot of the individual values of amoxicillin (µg/mL) recorded into piglets plasma. LLOQ: 10ng/ml; ULOQ: 10000ng/ml.

Nonetheless, when comparing the milk samples collected 2 hours after amoxicillin administration and the corresponding piglets' plasma, the difference seems to suggests that, at this time point, piglet exposure to drug in milk is low and not detectable with the developed method (**Figure E**). It is important to remember that piglet's plasma was collected 30 min after milk sampling to allow potential absorption in the bloodstream.

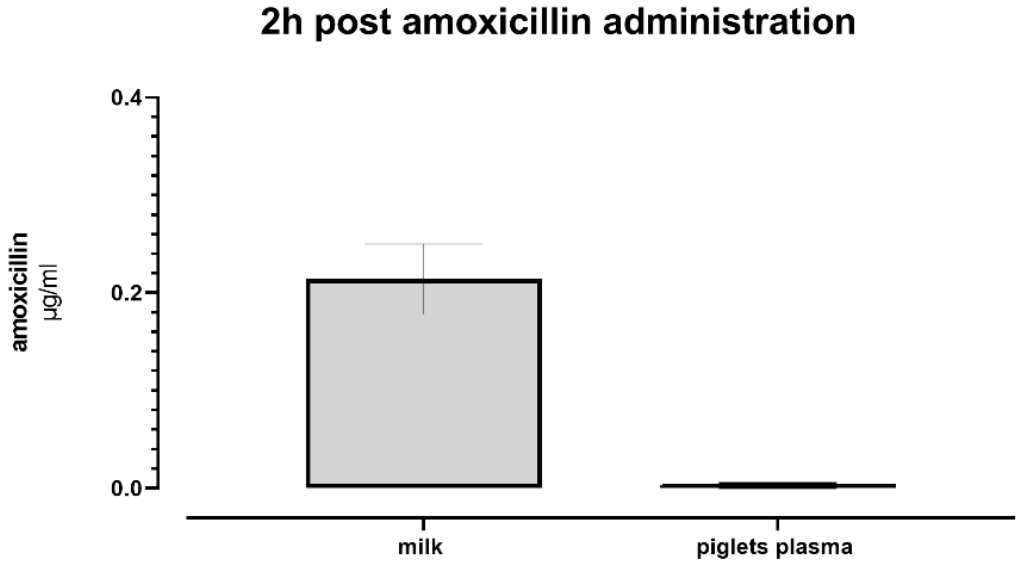

Figure E. Amoxicillin levels in sows' milk and piglets' plasma collected 2h after maternal dosing. Histograms represent the mean; error bars represent the SEM (standard error mean).

Discussion

The aim of this first lactation study was to assess if the swine species, selected upon extensive literature review (see deliverable D3.2), could effectively be used to build an *in vivo* lactation model able to provide quantitative and translatable data. Additionally, this preliminary study was necessary to assess the feasibility of the procedures, as little to no data are available with regards to milk collection in pigs. This preliminary study included conventional hybrid sows, that could never be used in the pharma setting for different reasons such as drug quantities and facility requirements, mainly due to the expertise and experience with these porcine breeds of the UNIBO research team. Despite the logistical challenges in working with animals weighing up to 300kg, large hybrid sows showed good maternal behavior, from time to time even excessive, to the point of showing aggression towards very well know trainers, especially in the first two weeks after delivery. However, conventional hybrid piglets are usually more numerous and bigger than Minipigs, thus blood collection was relatively easier and allowed for higher volumes of samples. Therefore, the UNIBO team performed the first trials on conventional sows, before moving to Göttingen Minipigs, to have a substantial feeling of what

was actually feasible looking at both the researchers' and the animals' points of view. Another "strength" of conventional sows was the bigger ears size, with many vessels, making for easier and faster catheter insertion procedures. Working with a "large" animal model species, the Göttingen Minipigs, allows for more extensive samplings, both in terms of volume and frequency of collection, leading to many future possibilities to further characterize the model itself. Pigs are relatively easy to train and get accustomed to human interaction in a short amount of time when the correct training procedures are applied; accordingly, sows were successfully clicker-trained with a food reward to be approached and touched on the ears (for blood sampling) and the abdominal region (for milk collection). The main refinement to the experimental procedures, along with the above-mentioned training, was the placement of a peripherally inserted central venous catheter (from an auricular vein) for repeated blood sampling. This allowed the researchers to collect blood samples without inflicting any pain to the sows while a second operator distracted them with a food reward. When looking at piglets' samplings, catheter placement was not feasible due to their size, but blood was collected under deep sedation achieved by Sevoflurane administered through a breathing mask. For every time point, only 2 piglets, selected in rotation within the litter, were sampled, allowing to minimize repeated sedation and stress. This is why individual identification of piglets was extremely important. For this preliminary trial, the research team decided not to collect any sample from the animals (sows and piglets) during the first week of lactation, to grant colostrum ingestion and maternal-offspring bonding and behavior. Despite this potentially representing a major flow to a "final" study design, as data regarding colostrum may be extremely important, it was important for this preliminary trial to start gradually and include procedures that seemed feasible based on the research team experience. Additionally, also ConcePTION WP4, working with human breastmilk, is avoiding colostrum to ensure that lactation is well established and minimize the differences in quantity of drugs in milk that would be linked to the differences in composition between colostrum and milk.

Amoxicillin was chosen as the "model" molecule for this preliminary trial for several reasons. First of all, it is one of the molecules that will be used by ConcePTION WP4 for a clinical study. Therefore, by the end of the project, the consortium will be able to compare *in vivo* data from both breastfeeding mothers and lactating sows. Additionally, amoxicillin is widely used for therapeutic purposes in porcine medicine, thus some background pharmacological data in the chosen species was already available. Such experiences allowed for a better prediction of potential side/adverse effects, deemed extremely low, thus giving the research team the chance to precisely analyse the ethical costs/benefits ratio and to focus on the study design and feasibility. The study design was technically feasible and well tolerated by the sows and the piglets. Animals did not show any discomfort-related behavior during the sampling procedures and the entire duration of the trial. Therefore, the research team will apply the same study design for the other model compounds to be tested (venlafaxine, levocetirizine and metformin), with some anticipated modifications in terms of timing of samples that will be tailored according to the existing PK/PD data in the porcine species.

As for the quantification of amoxicillin in the different matrices, the results are consistent between sows, even when comparing the different porcine breeds used. The plasmatic peak reached within 2 hours after IM administration is in line with existing literature. No background data were available for milk, yet the peak in its amoxicillin concentration was similar between animals and for the entire duration of the study (3 weeks). The lack of existing literature and data regarding infant exposure to amoxicillin through milk makes the interpretation of the piglets' results hard and only inferential. Indeed, the "absence" of amoxicillin in the vast majority of piglets' plasmatic samples may be explained by several factors potentially related to the study design and/or the analytic technique. From the study design point of view, now that data regarding milk concentrations are available, it looks like collecting piglets' blood at the 4h nursing may have provided different results. Therefore, this additional sampling time for piglets will be implemented in the amoxicillin trials that will be performed later in the project by the CRO Labcorp Drug Development (former COVANCE). Doing so will allow the research group to better understand the PK of amoxicillin transfer into milk and potential uptake into nursing piglets.

Conclusion

Overall, the study design used for this preliminary amoxicillin study has allowed to highlight both strengths and critical points of this new *in vivo* platform to test infant drug exposure through milk. The procedures were feasible and well tolerated by animals that gained confidence and trust during the preliminary training sessions. The availability of *in vivo* milk/plasma data will allow for detailed comparison with *in vitro* data generated for the same species, enabling to gain increased confidence in the *in vitro-in vivo* extrapolation (IVIVE) algorithms that can subsequently be translated to the human situation via PBPK modelling. The high-resolution of *in vivo* lactation data in Minipigs along with *in vitro* data obtained for the same model compounds in pig mammary epithelial cells (currently ongoing) will be instrumental to improve and refine generic templates and workflows for PBPK-based prediction of drug milk excretion in human.

3. Second step

As previously mentioned, in the early ‘60s two English biologists, named Russel and Burch, published “The Principle of Humane Experimental Technique” introducing the principles of the Three Rs: Replacement, Reduction and Refinement. That book marked the beginning of a new era of animal experimentation, were animal welfare assumed a primary role within European (and American) scientists. The Reduction concept refer to any strategy that will result in fewer animals being used to obtain sufficient data to answer the research question. One strategy to achieve Reduction is to maximize the informations obtained per experimental animal and thus potentially limiting (or avoiding) the subsequent enrollment of additional subjects.

It was in such regard that the UNIBO research team took advantage of the first *in vivo* trial by purchasing fecal samples of sows and piglets, without compromising animal welfare and the ConcePTION study itself. The aim of this collateral research was to evaluate the effect of sows’ milk, with amoxicillin residues, on the newborns gut microbiota, by means of an innovative *in vitro* piglet colon model. The Multi-Unit Colon Model : MICODE (Nissen et al. 2021) was used with piglets’ feces for a short-term colonic fermentation protocol of multiple sow’s milk samples, each containing different concentrations of amoxicillin residues in comparison to a blank control. The MICODE system allowed to mimic the setting of piglets’ gut ecology. Specifically, it was used to highlight the core microbiota shift, and the related volatilome, after colonic fermentation. To the authors’ knowledge, this study was the first evaluating the consequences of antibiotic residues with regards to the lactating piglets’ gut microbiota in an innovative *in vitro* colon model.

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Maternal amoxicillin affects piglets colon microbiota: microbial ecology and metabolomics in a gut model

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Abstract

The first weeks of life represent a crucial stage for microbial colonization of the piglets' gastrointestinal tract. Newborns' microbiota is unstable and easily subject to changes under stimuli or insults. Nonetheless, the administration of antibiotics to the sow is still considered as common practice in intensive farming for pathological conditions in the postpartum. Therefore, transfer of antibiotic residues through milk may occurs, affecting the piglets' colon microbiota. In this study, we aimed to extend the knowledge on antibiotic transfer through milk, employing an in vitro dedicated piglet colon model (MICODE—Multi Unit In vitro Colon Model). The authors' focus was set on the shifts of the piglets' microbiota composition microbiomics (16S r-DNA MiSeq and qPCR—quantitative polymerase chain reaction) and on the production of microbial metabolites (SPME GC/MS—solid phase micro-extraction gas chromatography/mass spectrometry) in response to milk with different concentrations of amoxicillin. The results showed an effective influence of amoxicillin in piglets' microbiota and metabolites production; however, without altering the overall biodiversity. The scenario is that of a limitation of pathogens and opportunistic taxa, e.g., *Staphylococcaceae* and *Enterobacteriaceae*, but also a limitation of commensal dominant *Lactobacillaceae*, a reduction in commensal *Ruminococcaceae* and a depletion in beneficial *Bifidobactericeae*. Lastly, an incremental growth of resistant species, such as *Enterococcaceae* or *Clostridiaceae*, was observed. To the authors' knowledge, this study is the first evaluating the impact of antibiotic residues towards the piglets' colon microbiota in an in vitro model, opening the way to include such approach in a pipeline of experiments where a reduced number of animals for testing is employed.

Key points

- *Piglet colon model to study antibiotic transfer through milk.*
- *MICODE resulted a robust and versatile in vitro gut model.*
- *Towards the "3Rs" Principles to replace, reduce and refine the use of animals used for scientific purposes (Directive 2010/63/UE).*

Keywords Antibiotic transfer · Antibiotic resistance · Swine reproduction · Volatilome · Microbiota · In vitro gut model

Andrea Gianotti and Maria Laura Bacci these authors share senior contribution.

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Introduction

In the last decades, the swine has been acknowledged as one of the most important preclinical species for a wide variety of physiological patterns. Indeed, the swine species show close similarities with humans, and the employment of pigs in research trials seems to be more widely accepted by society in terms of ethical values (Ventrella et al. 2021).

One of the latest interesting applications of this model is the study of transport of endogenous and exogenous molecules, such as pharmacological compounds, during the lactation stage which is possible via passive or active transport mechanisms, since the endothelium does not constitute a major barrier to solute movement between blood and the interstitium (Shennan and Peaker 2000). Transcellular transport requires solutes to cross the epithelial cell membranes, whereas paracellular transfer occurs between cells via leaky tight junctions (Shennan and Peaker 2000; Nauwelaerts et al. 2021). In particular, pharmacological compounds can reside in one (or more) milk fractions such as casein, fat globules, or free in the aqueous acid whey; it was acknowledged that hydrophilic drugs accumulate in the liquid medium (Ozdemir et al. 2018).

Since 2019, the European project entitled ConcePTION (n.d.) aims at generating accurate knowledge about the use of medication during pregnancy and breastfeeding (https://www.imi-conception.eu) by means of different approaches. Out of the latter, in vitro, in vivo, and in silico porcine trials have been established to generate data comparable and, most importantly translatable, to humans (Ventrella et al. 2019). Within said project, amoxicillin was chosen as the first test molecule since it is widely used for therapeutic purposes in both human and porcine medicine, with well-defined pharmacokinetics/pharmacodynamics (PK/PD) background data (Burch and Sperling 2018).

Amoxicillin is a bactericidal antibiotic in the group of aminopenicillins. When given orally to juvenile, but yet not suckling pigs, the bioavailability of amoxicillin varies between 25 and 31%, and thus, substantial drug quantities may have a direct impact on the gut microbiota (Burch and Sperling 2018). Indeed, swine gastrointestinal tract hosts a complex community of microorganisms, which compose the microbiota and take active part in immunity, digestive physiology, and nutrients metabolism (Luo et al. 2022). The microbiota of newborns is mainly transferred from the sow at birth and then later from the sow's colostrum and milk, but it is also shaped by the surrounding environment (Isaacson and Kim 2012; Luo et al. 2022). The microbiota of piglets is dominated by *Firmicutes*, and in particular by the orders *Lactobacillales* (Petri et al. 2010) and *Clostridiales* (Yang et al. 2021). The piglet's colon microbiota is inherited from the sow, not solely through milk, and among *Lactobacillales*, *Lactobacillaceae* early establish an important symbiosis that sculpture the intestinal epithelium up to the adult phase and bestow to the most beneficial effects derived from the microbiota (Petri et al. 2010).

In such regard, this study aims to evaluate the effect of sows' milk with different concentrations of amoxicillin, widely used as antibiotic in piggeries, on the perturbations of the newborns gut microbiota using an innovative in vitro colon model. We used multi-unit in vitro colon model: MICODE (Nissen et al. 2021a, b) modified by using piglets' feces from four healthy animals for a short-term colonic fermentation protocol (24 h) of different sow's milk containing different concentrations of amoxicillin residues in comparison to a sow's milk with no antibiotic and to a blank control. This system permitted to resemble in vitro the in vivo conditions of piglets' gut ecology, in line with the international call to reduce animal testing (Directive 2010/63/EU; Regulation (EU) 2019/1010). In particular, it serves to highlight the shift that happens in the core microbiota and in the related volatilome after colonic fermentation. The results were obtained coupling microbiomics (qPCR and 16S-rDNA MiSeq) and metabolomics (SPME GC–MS) and studying several ecological indicators either related to microbes and molecules, as follows: (i) microbial biodiversity, (ii) microbial eubiosis, (iii) shifts in the core microbiota at high or low taxonomical levels of selected opportunistic and beneficial commensals taxa, (iv) production of postbiotics, (v) production of detrimental compounds.

Materials and methods

Preparative

Conventional pregnant sows were purchased from a local farm, SUIMAX di Massimo Ferri (Via San Michele 718, Valsamoggia 40,056 BO, Italy), chosen of the basis of the microbiological status of the facility and for the reproductive track records. All piglets included in the study were born from the abovementioned sows in the experimental facility of the ANFI-ASA Unit, Department of Veterinary Medical Sciences, Alma Mater Studiorum—University of Bologna (via Tolara di Sopra 50, Ozzano dell'Emilia 40,064 BO, Italy). Sows were transferred to the experimental facility 1 month prior the expected delivery date and moved to the farrowing pen one week before. Animals were fed a standard pellet formula specifically made for breeding animals, produced by a local vendor (Molini Popolari Riuniti, Ellera-Umbertide 6019 PG, Italy). Drinking water was provided ad libitum, while the daily feed ratio was divided into two portions: early morning and afternoon. Light/dark cycle was set at 12/12 h with a min of 40 lx during light hours.

Temperature was set at 21 ± 1 °C to meet sows thermal needs. With regards to piglet, two heat lamps were placed in dedicated areas of the farrowing crate to reach 32 ± 1 °C. For this study, only animals previously enrolled in an experimental protocol approved by the Local Ethics Committee and the Italian Ministry of Health were used (Legislative Decree 26/2014, authorization n° 32/2021-PR, protocol number 2216A.17). The abovementioned experimental protocol already included samplings on sows and piglets.

Briefly, fecal samples from piglets were collected, processed and used as the representation of the piglets' colon microbiota to undergo colonic short-term in vitro batch fermentation of sows' milk with different residues of amoxicillin in comparison to another antibiotic free milk sample.

Piglets' fecal samples

Fecal samples were obtained from four 7-day-old piglets, maintained refrigerated, and processed within few hours. The fecal slurry was prepared by homogenizing 8 g of pooled feces (2 g of each piglet) in 72 mL of pre-reduced phosphate-buffered saline (PBS).

Sows milk (treatment and control samples)

Amoxicillin (Clamoxyl® RTU, Pfizer, New York, NY) was administered to sows, SID (standardized ileal digestible) at 7 mg/kg IM (intramuscular) from the second week of lactation until weaning (day 28). Milk samplings were manually obtained at different timepoints, after a prior administration of exogenous oxytocin, and immediately frozen (− 80 °C) to preserve amoxicillin and its metabolites. Three kinds of milk employed in the in vitro fermentation experiments were obtained from two pluriparous conventional adult sows aged two and three years approximately. Amox07 and amox08 are milk samplings from the first sow, with different concentrations of amoxicillin, collected 24 h and 2 h post intramuscular administration respectively, 9 days from the onset of lactation. Amox02 is the milk sample with no amoxicillin residues from the second lactating sow, used as the positive control and collected 6 days post-parturition. The blank control was instead used as a negative control. Milk samples were stored at − 80 °C and analyzed at the bioanalytical laboratory of BioNotus® (Niel, Belgium) using a validated liquid chromatography–mass spectrometry (LC–MS/MS) method (BioNotus Method: MT-500A). Analyses were performed using Shimadzu Nexera X2 UHPLC, coupled with Shimadzu LC–MS 8050 system (Shimadzu, Kyoto, Japan). The data was acquired and processed via LabSolutions version 6.81 software (Shimadzu). The lower and upper limit of quantification of amoxicillin were 10 ng/mL and 10,000 ng/mL respectively.

Fecal batch-culture fermentation and sample collection

Colonic fermentations were conducted for 24 h in independent vessels on 1% (w/v) of amox02, on 1% (w/v) of amox07, on 1% (w/v) of amox08 (positive control), and on a blank control (BC) (negative control), using the in vitro gut model MICODE, obtained by the assembly of Minibio Reactors (Applikon Biotechnology BV, Delft, NL) and controlled by Lucullus PIMS software (Applikon Biotechnology BV, NL) (Nissen et al. 2021a, b). The preparation of the experiments was made according to published procedures (Koutsos et al. 2017; Wang et al. 2020; Nissen et al. 2021a, b; Venardou et al. 2021). In details, bioreactors were autoclaved at 121 °C and 100 kPa for 15 min and once cooled aseptically filled with 90 mL of anaerobic pre-sterilized fermentation medium (FM) (Venardou et al. 2021). FM contained (per liter): 5 g/L yeast extract, 10 g/L ascorbic acid, 10 g/L sodium acetate, 5 g/L $(NH_4)_2SO_4$, 2 g/L urea, 0.2 g/L $MgSO_47H_2O$, 0.01 g/L $FeSO_47H_2O$, 0.007 g/L $MnSO_4xH_2O$, 0.01 g/L NaCl, 1 ml/L Tween 80, 0.05 g/L hemin, and 0.5 g/L L-cysteine hydrochloride. The pH was adjusted to 7.0. Fermentation vessels were filled aseptically with 90 mL of FM and the bioreactor headplates were mounted, including previously sterilized and calibrated sensors, i.e., pH and DO_2 (dissolved oxygen) sensors (AppliSense, Applikon Biotechnology BV, NL). Anaerobic condition (0.0–0.1% w/v of DO_2) in each bioreactor was obtained in about 30 min flushing with filtered O_2-free N_2 through the mounted-in sparger of Minibio reactors (Applikon Biotechnology BV, NL), and was constantly kept over the experiment. Temperature was set at 39 °C and stirring at 100 rpm, while pH was adjusted to 7.0 and kept throughout the experiment with the automatic addition of filtered NaOH or HCI (0.5 M). Once the exact environmental settings were reached, each of the four vessels was aseptically injected with 10 mL of pooled fecal slurry (10% w/v of pooled piglets' feces to a final concentration of 1%, w/v) and then three of them independently with 1 mL of amox02, amox07, or amox08 (to a final concentration of 1%, w/v), while the fourth vessel was set as blank control (BC, basal medium and 1% fecal slurry only). Batch cultures were run under these controlled conditions for a period of 26.10 h during which samples were collected at 3 time points (BL, baseline; T1 = 18 h; and EP = 24 h). Baseline (BL) was defined on the first pH changes (Venema 2015) detected by Lucullus (1 read/10 s) via the pH Sensors of MICODE (AppliSense Sensors, Applikon Biotechnology BV, NL). For this work, the BL was set after 2.10 ± 0.28 h. Sampling was performed with a dedicated double syringe–filtered system (Applikon Biotechnology BV, NL) connected to a float drawing from the bottom of the vessels without perturbing or interacting with the bioreactor's ecosystem. To guarantee a close control, monitoring, and recording of fermentation parameters, the software Lucullus 3.1 (PIMS, Applikon Biotechnology

BV, Delft, NL) was used. This also allowed to keep the stability of all settings during the experiment. Fermentations were conducted in duplicate independent experiments, using for the first the fresh pooled slurry in pre-reduced PBS and for the second the same pooled slurry in pre-reduced PBS and 15% glycerol, previously stored at − 80 °C for a week (Asare et al. 2021).

Experimental set up and pipeline of activities

Parallel and independent vessels for amox02, amox07, amox08, and blank control were run for 24 h after the adaptation of the fecal inoculum, defined as the baseline (BL). The entire experiment consisted of 9 duplicated biological cases ($n = 18$), including 4 theses (amox02, amox07, amox08, and BC) and 3 time points (BL = 2.10 h, T1 = 18 h, and EP = 24 h) in duplicate. Samples of the different time points were used for qPCR and SPME GC–MS analyses. Pooled samples at the BL and the EPs of the 4 fermentation theses were used for 16S-rDNA MiSeq analyses (Illumina Inc, San Diego, CA, USA). After sterile sampling of 6 mL of bioreactor contents, samples were centrifuged at 16,000 × g for 7 min to separate the pellets and the supernatants, which were used for bacterial DNA extraction and SPME–GC–MS analysis, respectively (Nissen et al. 2021a, b). Specifically, microbial DNA extraction was conducted just after sampling so as not to reduce *Firmicutes* content (Nissen et al. 2021a, b). DNA samples and solid phase micro-extraction (SPME) GC–MS samples were then stored at − 80 °C. Technical replicates of analyses were conducted in duplicate for SPME GC–MS ($n = 36$), in triplicate for qPCR ($n = 54$), and in single pooled cases ($n = 5$) for MiSeq.

Microbiomics

DNA extraction

Bacterial DNA was extracted from the MICODE eluates at each time points, just after sampling; at the baseline (BL, when the fecal inoculum adapted to the in vitro condition), at the intermediate time point (T1, after 18 h), and at the endpoint (EP, after 24 h) using the Purelink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Bacterial DNA was extracted also from frozen sow's milk using the NucleoSpin Food DNA Isolation Kit (Macherey–Nagel, Duren, De). Nucleic acid purity and concentration was tested on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK).

DNA amplification and sequencing by Illumina MiSeq

Samples from the BL and the EP were used for MiSeq sequencing (Illumina Inc, USA). Bacterial diversity was obtained by the library preparation and sequencing of the 16S rRNA gene. The following two amplification steps were performed: an initial PCR amplification using 16S locus-specific PCR primers (16S-341F 5′-CCTACGGGNGGC WGCAG-3′ and 16S-805R 5′-GACTACHVGGGTATCTA ATCC-3′) and a subsequent amplification integrating relevant flow-cell-binding domains (5′-TCGTCG GCAGCG TCAGATGTGTATAAGAGACAG-3′ for the forward primer and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAG-3′ for the reverse overhang), and lastly unique indices selected among those available Nextera XT Index Kits were combined according to manufacturer's instructions (Illumina Inc, USA). Both input and final libraries were quantified by Qubit 2.0 Fluorometer (Invitrogen, USA). In addition, libraries were quality-tested by Agilent 2100 Bioanalyzer High Sensitivity DNA assay (Agilent technologies, Santa Clara, CA, USA). Libraries were sequenced in a MiSeq (Illumina Inc, USA) in the paired end with 300-bp read length (Marino et al. 2019). Sequencing was conducted by IGA Technology Service Srl (Udine, Italy).

Sequence data analysis

Reads were de-multiplexed based on Illumina indexing system, as described in Marino et al. (2019). Sequences were analyzed using QIIME 2.0 (Caporaso et al. 2010). After filtering based on read quality and length (minimum quality = 25 and minimum length = 200), operational taxonomic units (OTUs) defined by a 97% of similarity were picked using the Uclust v1.2.22 q method (Edgar 2010), and the representative sequences were submitted to the RDP classifier (Wang et al. 2007) to obtain the taxonomy assignment and the relative abundance of each OTU using the Greengenes 16S rRNA gene database (Version 2013_8) (McDonald et al. 2012). Alpha and beta diversity analyses were performed using QIIME 2.0.

Absolute enumeration of bacterial groups by qPCR

Enumeration of bacterial groups was made by qPCR to quantify the microbiota at the BL and evidence changes after fermentation (Tanner et al. 2014; Westfall et al. 2018; Tsitko et al. 2019; Tamargo et al. 2022) and from the milk samples to quantify the bacterial loads, following previous protocols (Modesto et al. 2011; Nissen et al. 2021a, b). For milk samples, 8 bacterial taxa were analyzed, namely *Eubacteria*, *Firmicutes, Lactobacillales*, *Bifidobacteriaceae*, *Enterobacteriaceae*, *Clostridium* group I, *Clostridium* group IV, and *Escherichia coli.* For colonic fermentation samples, the previous 8 and other 6 taxa were analyzed, namely *Bacteroidetes*, *Bacteroides-Prevotella-Porphyromonas* (BPP) group, *Atopobium-Collinsella-Eggerthella* (ATOP) group, *Bifidobacterium longum*, *Faecalibacterium prausnitzii*, and

Akkermansia muciniphila) (Supplemental Table S1) were assessed by qPCR on a QuantStudio 5 System (Applied Biosystem, Thermo Fisher, USA).

Metabolomics

Volatilome analysis

Volatile organic compound (VOCs) evaluation was carried out on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent Technologies 5975 mass spectrometer operating in the electron impact mode (ionization voltage of 70 eV) equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, the Netherlands). The SPME GC–MS protocol and the identification of volatile compounds were done according to previous reports, with minor modifications (Guerzoni et al. 2007; Di Cagno et al. 2011; Casciano et al. 2021; Nissen et al. 2021a). Briefly, 3 mL of vessel content were centrifuged at 16,000 × g for 7 min at 4 °C and then the supernatant placed into 10-mL glass vials containing 10 µL of the internal standard (4-methyl-2-pentanol) to a final concentration of 4 mg/L. Samples were then equilibrated for 10 min at 45 °C. SPME fiber, coated with carboxen-polydimethylsiloxane (85 µm), was exposed to each sample for 40 min. Preconditioning, absorption, and desorption phases of SPME–GC analysis, and all data-processing procedures were carried out according to previous publications (Di Cagno et al. 2011; Casciano et al. 2021; Nissen et al. 2021a). Briefly, before each head space sampling, the fiber was exposed to the GC inlet for 10 min for thermal desorption at 250 °C in a blank sample. The samples were then equilibrated for 10 min at 40 °C. The SPME fiber was exposed to each sample for 40 min, and finally the fiber was inserted into the injection port of the GC for a 10-min sample desorption. The temperature program was 50 °C for 1 min, then programmed at 1.5 °C/min to 65 °C, and finally at 3.5 °C/min to 220 °C, which was maintained for 25 min. Injector, interface, and ion source temperatures were 250, 250, and 230 °C, respectively. Injections were carried out in split-less mode and helium (3 mL/min) was used as a carrier gas. Identification of molecules was carried out by searching mass spectra in the available databases (NIST 11 MSMS library and the NIST MS Search program 2.0 (NIST, Gaithersburg, MD, USA). Each VOC was relatively quantified in percentage (limit of detection, LOD = 0.001 mg/kg) (Bonfrate et al. 2020).

Shift of main microbial VOCs

In samples prior to in vitro colonic fermentation (BL) (Supplemental Table S2), the main microbial metabolites related to fermentation of foods were also absolutely quantified in mg/kg with the aforementioned SPME GC–MS approach and the internal standard, but with different cutoffs: LOQ (limit of quantification) = 0.03 mg/kg and LOD = 0.01 mg/kg) (Di Cagno et al. 2011; Casciano et al. 2021; Nissen et al. 2021a). For these compounds, samples at T1 and EP were compared to the BL and values were expressed as shifts. Values were computed as follows: (i) each single compound was normalized (mean centering method) within its dataset, which included cases from amox02, amox07, and amox08, and the blank control at different time points; (ii) the BL dataset (Supplemental Table S2) was then subtracted to the fermentation time points; (iii) post hoc analysis was done to compare the sample productions of a single molecule.

Data processing and statistical analysis

For metabolomics, one-way ANOVA model ($p < 0.05$) was used to determine significant VOCs among the raw data of peak's area of the GC–MS chromatograms. The significant VOCs ($n = 65$) representing the total volatilome of the experiments were analyzed differently: (i) 8 main VOCs related to microbial fermentation of foods were absolutely quantified and normalized and their BL values were subtracted from T1 and EP values and represented as blox plots, including post hoc Tukey HSD test ($p < 0.05$); (ii) the remaining volatilome was relatively quantified, sorted for main chemical classes and super-normalized, then each dataset was computed for principal component analysis (PCA) to distribute the results on a plane and coupled to multivariate ANOVA (MANOVA) ($p < 0.01$) to address specific contributes by categorical predictors.

For the sequencing data analysis, the QIIME pipeline version 2.0 was used. Within-community diversity (alpha diversity) was calculated using observed OTUs, Chao1 Shannon, Simpson, and Good's coverage indexes with 10 sampling repetitions at each sampling depth. Student's *t*-test was applied to compare the latest sequence/sample values of different treatments within an index. Analysis of similarity (ANOSIM) and the ADONIS test were used to determine statistical differences between samples (beta diversity) following the QIIME compare_categories.py script and using weighted and unweighted phylogenetic UniFrac distance matrices. Principal coordinate analysis (PCoA) plots were generated using the QIIME beta diversity plots workflow (Marino et al. 2019).

For microbiomics, ANOVA model for group comparison (BL versus EPs) ($p < 0.05$) was performed for MiSeq and MANOVA ($p < 0.05$) model (categorized for the time points and the treatments) was performed for qPCR. Afterwards, the significant variables and others of peculiar interest were selected and the shifts in abundance were calculated as Log_2(F/C) (Love et al. 2014). Then, post hoc Tukey HSD test on the raw data ($p < 0.05$) was performed to define

differences among treatments (MiSeq and qPCR) or time points (qPCR). The baselines of values for the volatilome and for the microbiota were that obtained sampling just after adaptation of the microbiota to the bioreactor condition (Nissen et al. 2021b). Normalization of datasets was performed with the mean centering method. Statistics and graphics were made with Statistica v.8.0 (Tibco, Palo Alto, CA, USA).

The NCBI Bioproject PRJNA862673 is available at https://www.ncbi.nlm.nih.gov/bioproject/862673 including Biosamples and relative SRAs, which will be release at least 2022–12-15, or with the release of linked data, whichever is first.

Results

Amoxicillin LC–MS/MS quantifications

Amox07 milk sample was collected 24 h post administration; amoxicillin was found below limit of quantification (i.e., < 10 ng/mL). Instead, amox08, collected 2 h post maternal administration, was quantified as 32.741 ng amoxicillin/mL. Amox02 was not analyzed as the sow was never treated with amoxicillin; this sample was used as positive control.

Microbiomics

Analysis of the biodiversity in the microbiota by relative quantification of 16S-rDNA

The microbiota diversity indices were analyzed both to study the impact of different amoxicillin residues in the sow's milk on microbial population of piglets' colon and to assess population's stability during fermentation of the different bioreactors (Supplemental Fig. S1). The BL value (as defined by first pH decrease) was compared to the EPs of fermentation of different treatments. Considering richness, it is unquestionable that an increase (observed OTUs) cannot happen during in vitro fermentation (Isenring et al. 2021), and reductions in respect to the BL were significantly different just for EPs of amox02 and amox07. A reduction in abundance index (Chao 1) from the BL to the EP was recorded for amox02 and amox07, while amox08 scored a slight increase, although significant differences were just that of the highest values (amox08) in respect to the lowest values (amox07). Significant reduction in evenness (Shannon) from the BL to the EP were seen for any substrate, and different values were recorded at the EP of amox08 in respect to the lowest values of amox07. This latter feature could be a first clue to a possible perturbation of microbiota eubiosis. Reductions in dominance (Simpson) were seen from the BL to the EP for any substrate, but significantly just for amox07. This latter feature could be ascribed to the reduction at the EP of a dominant phylum. Additionally, the Good's index, relative to rare OTUs, was kept similar from the BL to the EPs of any milk substrate with just slight reductions, but no significative differences. This feature means that the stability of MICODE environment was maintained throughout the entire experimental period, because the rare taxa, which need strict ecological conditions, were still present at the EPs. When the bacterial diversity between samples (beta diversity) was examined with Bray–Curtis analysis, the pooled sample relative to the BL was set not so much distant, although discriminated in respect to the samples at the EP of fermentation, as demonstrated by principal coordinate analysis (PCoA) based on an unweighted (qualitative) phylogenetic UniFrac distance matrix.

Analysis of the shift in the phyla of microbiota by relative quantification of 16S-rDNA

Results from microbiota analyses at the phylum level (Table 1) have defined that the core microbiota of any sample was ruled by two main phyla with relative abundance higher than 10%, and three minors with relative abundance lower than 10%. *Firmicutes* and *Bacteroidetes*, accounted for almost the 80% of the whole pie, while *Actinobacteria*, *Proteobacteria*, and *Fusobacteria* accounted for the remaining. In any fermentation sample, *Firmicutes* and *Bacteroidetes* were reduced in respect to the BL, although not significantly. *Actinobacteria* were reduced significantly in any milk fermentations, while *Fusobacteria* and *Proteobacteria* were increased, but significantly just for the latter. The unaffected changes of the core microbiota make us generally believe that an equilibrium among such wide taxa was maintained even after fermentation.

Table 1 Shifts of the microbiota at the phylum level from 16S-rDNA sequencing

#OTU ID	% R.Q	Log_2(F/C)			ANOVA*
	Baseline	amox02	amox07	amox08	*p* value
Euryarchaeota	0.01^{a}	-2.50^{b}	-3.03^{b}	-2.56^{b}	0.001626
Bacteria; Other	0.04^{a}	-2.00^{b}	-1.99^{b}	-1.35^{b}	0.017493
Actinobacteria	2.71^{a}	-2.95^{b}	-2.64^{b}	-1.59^{b}	0.024128
Bacteroidetes	21.66	−1.18	−1.08	−2.56	0.080744
Firmicutes	61.69	−0.81	−0.41	−0.32	0.173732
Fusobacteria	8.12^{a}	2.09^{b}	1.91^{b}	1.99^{b}	0.009156
Proteobacteria	5.75	1.83	1.11	1.24	0.189654
Synergistetes	0.02^{a}	-3.76^{b}	0.00^{a}	-3.24^{b}	0.004404

*One-way ANOVA with $p < 0.05$. *R.Q.*, relative quantity. [abc]Letters indicate significant differences within a line by Tukey's honestly significant differences (HSD) test ($p < 0.05$)

Analysis of the shift in the families of microbiota by relative quantification of 16S-rDNA

Results from the microbiota analysis at the family level (Table 2) evidenced a scenario discriminated by the fermentation and seldom by the severity of amoxicillin concentration.

Indeed, amox08 during colonic fermentation was able to reduce the content of opportunistic *Porhyromonadaceae* and limit the growth of *Staphylococcaceae*, *Enterobacteriaceae*, and *Desulfovibrionaceae* in a significative difference in respect to the milk control with no antibiotic residues (amox02). Oppositely, the antibiotic residues exerted an undesired effect towards important beneficial taxa of the piglets' colon microbiota, due to a wider range of targets. This effect was different in respect to the different capacity of a taxon to generically resist to insults. In particular, this effect was dramatically high in sensitive *Bifidobacteriaceae*, which were almost depleted after amox08 fermentation, and in sensitive *Ruminococcaceae*, which were reduced of almost three-folds, in respect to the BL and two time more than the milk without antibiotic residues. Also, this effect was observed in dominant *Lactobacillaceae*. Unexpectedly, this effect was observed also for important commensal fibrolytic bacteria, such as *Bacteroidaceae*, that was reduced of 2.4-folds in respect to the BL, although not significantly. Furthermore, it is observed in some taxa a competitive advantage by the presence of antibiotic residues, recording an increased abundance. This phenomenon was particularly strong in those bacterial taxa phenotypically heterogeneous. For example, from the superior taxonomic level of *Lactobacillales*, two family behaved oppositely; as we have just said, the *Lactobacillaceae* were reduced (from 34.5% at the baseline to 7.6% at the endpoint of fermentation with amox08), but the *Enterococcaceae* were fostered (from 0.16% at the baseline to 14.5% at the endpoint of fermentation with amox08). Similarly, from the superior level of *Gammaproteobacteria*, the *Enterobacteriaceae* were more limited (from 4.4% at the baseline to 19.5% and 10.0% at the endpoint of amox02 and amox08 fermentations, respectively), but the *Pasteurellaceae* were increased (from 0.07% at the baseline to 0.6% and 3.3% at the endpoint of amox02 and amox08 fermentations, respectively). We can summarize the presence of antibiotic residues in the milk can modulate the microbiota of piglets via four main actions. (i) A desired inhibitory effect towards several opportunistic bacterial taxa; (ii) an inhibitory effect towards sensitive commensal taxa; (iii) a stimulation of tough (generally resistant to stress) bacterial taxa.

Table 2 Shifts of the microbiota at the family level from 16S-rDNA sequencing

#OTU ID	% R.Q	Log_2(F/C)			ANOVA*
	M11 BL	amox02	amox07	amox08	*p* value
Actinomycetaceae	2.55[a]	−3.11[b]	−2.61[b]	−1.61[b]	0.024667
Bifidobacteriaceae	0.06 [a]	−2.34[b]	−3.10[b]	−3.89[b]	0.001018
Bacteroidaceae	18.48	−0.99	−0.88	−2.41	0.120363
Porphyromonadaceae	1.30[a]	−3.04[b]	−3.11[b]	−4.22[b]	0.002328
Prevotellaceae	0.83[a]	−5.71[b]	−5.24[b]	−5.85[b]	0.000033
Rikenellaceae	0.26[a]	−7.31[b]	−7.84[b]	−6.78[b]	0.000008
Sphingobacteriaceae	0.72[a]	−3.74[b]	−3.86[b]	−2.91[b]	0.002006
Staphylococcaceae	0.01[b]	1.34[a]	1.19[a]	0.42[ab]	0.000149
Enterococcaceae	0.17[b]	5.48[a]	5.86[b]	6.43[b]	0.027770
Lactobacillaceae	34.52[a]	−1.89[b]	−3.14[b]	−2.19[b]	0.010708
Streptococcaceae	0.93[a]	−2.08[b]	−2.45[b]	−1.51[b]	0.017605
Clostridiales; other	0.09[a]	−3.73[b]	−5.26[b]	−4.79[b]	0.000991
Clostridiaceae	2.44	1.84	3.52	2.21	0.057749
Lachnospiraceae	9.30[a]	−2.18[b]	−2.59[b]	−2.29[b]	0.001559
Peptococcaceae	0.66	−0.53	−0.84	−0.60	0.096887
Peptostreptococcaceae	2.20[a]	−0.75[b]	−1.55[c]	0.25[b]	0.000012
Ruminococcaceae	7.04[a]	−2.73[b]	−1.37[b]	−2.93[b]	0.002811
Veillonellaceae	2.13	1.19	−1.85	1.91	0.638170
Coriobacteriaceae	0.34	−1.02	0.61	0.66	0.805605
Coprobacillaceae	0.26[b]	0.00[b]	−0.05[b]	1.68[a]	0.000092
Erysipelotrichaceae	1.52	−1.84	−2.51	−1.76	0.009737
Fusobacteriaceae	8.12[a]	2.09[b]	1.91[b]	1.99[b]	0.009156
Alcaligenaceae	0.26[a]	−0.39[a]	−2.12[b]	−1.53[b]	0.045315
Desulfovibrionaceae	0.66	−4.72	−5.25	−5.17	0.000032
Campylobacteraceae	0.01	1.83	2.17	1.50	0.113515
Enterobacteriaceae	4.40	2.15	1.38	1.19	0.256852
Pasteurellaceae	0.08[b]	3.01[a]	3.42[a]	5.46[a]	0.023245

*One-way ANOVA with $p < 0.05$. *R.Q.*, relative quantity. [abc]Letters indicate significant differences within a line by Tukey's honestly significant differences (HSD) test ($p < 0.05$)

Analysis of the shift in the genera and species of microbiota by relative quantification of 16S-rDNA

In order to try to account the shift previously observed to

some specific taxa, a relative quantification of 16S-rDNA was performed (Table 3). Specifically, the reduction of *Lactobacillaceae* in contrast to the increase in *Enterococcaceae* has been generated by some key players, as *Lactobacillus crispatus* (from 9.7% at the baseline to 2.2% and 1.0% at the endpoint of amox02 and amox08 fermentations, respectively), *Lactobacillus antrii* (from 8.3% at the baseline to 3.7% and 3.6% at the endpoint of amox02 and amox08 fermentations, respectively), *Lactobacillus gasseri* (from 9.5% at the baseline to 1.6% and 1.4% at the endpoint of amox02 and amox08 fermentations, respectively), and *Lactobacillus delbruecki* (from 1.5% at the baseline to 0.3% and 0.1% at the endpoint of amox02 and amox08 fermentations, respectively). Oppositely, under the *Enterococcaceae*, the species that were overrepresented were *Enterococcus durans* (from 0.1% at the baseline to 3.2% and 4.5% at the endpoint of amox02 and amox08 fermentations, respectively) and *Enterococcus faecalis* (from cutoff levels at the baseline to 3.7% and 9.0% at the endpoint of amox02 and amox08 fermentations, respectively).

For the intestinal health of piglets, the role of *Clostridiales* is crucial, because represents a large portion of the core microbiota (Yang et al. 2021). Actually, our samples accounted for about the 24% of total microbiota at the baseline. They include some pathogen targets of amoxicillin, but others are commensals butyrate producers. For example, while the stress sensitive *Lachnospiraceae* or the *Ruminococcaceae* were around tenfold inhibited in amox08 colon-fermented microbiota in comparison to the control, with the same milk the opportunistic *Veillonaceae* and *Peptostreptococcaceae* slightly increased and even more in *Clostridiaceae* (fivefold). In particular, within this latter family, another phenotypical split had happened, indeed even if all the three major genera of this family were fostered by any milk substrate, the sole genus *Clostridium* grew less than the control (7.6% and 6.9% at the endpoint of amox08 and amox02 fermentations, respectively), while genera *Finegoldia* (0.7% and 2.5% at the endpoint of amox02 and amox08 fermentations, respectively) and *Anaerococcus* (0.3% and 1.7% at the endpoint of amox02 and amox08 fermentations, respectively) were increased much more than the control. Noteworthy, even deeper in the genus *Clostridium*, some species were limited while others were fostered after fermentation with amox08. For example, the harmful *Clostridium perfringens* (from 1.9% at the baseline to 1.4% at the endpoint), *Clostridium baratii*, and *Clostridium frigidicarnis* were underrepresented, while *Clostridium butyricum* and *Clostridium cadaveris* were overrepresented.

Absolute enumeration of selected taxa of milk

We firstly considered milk microbiota to give a more complete picture of all the ecological factors affecting microbial shift in MICODE gut model. For a robust description of the core microbiota and its shifts produced after fermentation of the different milk samples, we performed qPCR absolute quantifications of 10 selected targets related to healthy piglets' colon ecology, either at top or low taxonomic levels. We have also considered the bacterial loads of 8 principal bacterial taxa common in sow's milk. Considering milk, generally there were significant differences mainly comparing the milk samples with no antibiotic residues (amox02) or the milk samples with the lowest antibiotic residues (amox07) to the milk samples with antibiotic residues (amox08). In the milk samples, total bacterial load accounted for a mean of 1.12E + 06 cells/mL and amox08 had 44% significantly less abundance than the milk with no antibiotic residues. *Firmicutes* content had a mean value of 2.78E + 05 and amox08 had 40% significantly less abundance than amox02. *Lactobacillales* content had a mean value of 2.01E + 05 and amox08 had 40% and 33% significantly less abundance than amox02 and amox07, respectively. *Clostridium* group I and *Clostridium* group IV had means values of 1.91E + 04 and 2.59E + 04, and amox08 had 32% and 46% significantly less abundance than amox02, respectively. *Enterobacteriaceae* had a mean value of 1.41E + 04 and amox08 had 34% significantly less abundance than amox02. In this family, *Escherichia coli* was detectable just in the amox02 and amox07 samples, accounting for a mean value of 1.68E + 02. A similar outcome was also seen for the content of *Bifidobacteriaceae* that was detectable just in the amox02 and amox07 samples, accounting for a mean value of 1.8E + 04. From these results, it is possible to summarize that the presence of amoxicillin residues in the milk diminished depending on concentration its indigenouos microflora.

Absolute enumeration of selected taxa of colonic fermentation samples

With the same analytical approach, the shifts occurred during MICODE fermentation were considered. In general, significant differences were found for the milk substrates, but not for the blank control. At the BL, the abundance similarly averaged (no significant differences among BL raw data) for 1.05E + 10 and trended to increase, except for the blank control, with no significant differences (Table 4). Considering the two main phyla, in fermentation samples, *Firmicutes* and *Bacteroidetes* had opposite trends. The former was increased by amox02 and amox07 and reduced by amox08 (of about 2.62E + 09 cells/mL), the latter was reduced by each milk samples, but not significantly for amox02. In particular, amox08 reduced *Bacteroidetes* of about 1.39E + 09 cells/mL, which was circa 9 time more the reduction of amox02. In the taxon *Firmicutes*, the *Lactobacillales* recorded an increase for amox02 and significant reduction just for amox08, which was reduced almost thrice

Table 3 Shifts of the microbiota at the genus and species level from 16S-rDNA sequencing

#OTU ID	% R.Q	Log_2(F/C)			ANOVA*
	Baseline	amox02	amox07	amox08	*p* value
Methanobrevibacter	0.01[a]	−2.50[b]	−3.03[b]	−2.56[b]	0.001626
Actinomyces	2.54[a]	−3.10[b]	−2.60[b]	−1.61[b]	0.024542
Corynebacterium	0.02	−0.68	−2.21	−0.24	0.360138
Bifidobacterium	0.06[a]	−3.34[b]	−3.10[b]	−3.89[b]	0.001018
Bacteroides	18.48[a]	−0.99[b]	−0.88[ab]	−2.41[c]	0.017239
Porphyromonas	0.09[a]	−4.50[b]	−6.35[b]	−5.29[b]	0.000364
Parabacteroides	1.21[a]	−2.97[b]	−3.01[b]	−4.17[b]	0.002679
Prevotella	0.83[a]	−5.71[b]	−5.24[b]	−5.85[b]	0.000033
Rikenella	0.26[a]	−7.31[b]	−7.84[b]	−6.78[b]	0.000008
Enterococcus	0.17	5.48	5.86	6.43	0.127750
Lactobacillus	34.52[a]	−1.99[b]	−3.14[b]	−2.19[b]	0.010708
Streptococcus	0.93[a]	−2.08[b]	−2.45[b]	−1.51[b]	0.017605
Clostridiaceae; other	0.23[a]	−3.73[b]	−5.26[b]	−4.79[b]	0.000991
Clostridium	2.07	1.88	3.52	1.74	0.434835
Finegoldia	0.01	6.09	8.24	7.94	0.284612
Mogibacterium	0.21[a]	−4.52[b]	−4.19[b]	−3.29[b]	0.001492
Lachnospiraceae; other	2.61[a]	−1.74[b]	−3.10[b]	−2.22[b]	0.017499
Blautia	0.03	−1.92	−0.59	−0.74	0.185554
Dorea	1.02[a]	−3.11[b]	−4.92[b]	−3.22[b]	0.003279
Roseburia	0.01	−0.44	−0.70	−0.24	0.189321
Ruminococcus	5.59[a]	−2.41[b]	−3.38[b]	−2.24[b]	0.007038
Peptococcus	0.66	−0.54	−0.84	−0.60	0.056183
Peptostreptococcaceae; other	0.09	2.98	2.14	4.27	0.399730
Clostridium	0.31[a]	−1.73[bc]	−2.28[c]	−1.08[b]	0.049276
Peptostreptococcus	1.79[a]	−1.92[b]	−2.73[b]	−1.45[b]	0.026758
Faecalibacterium	0.47[a]	−2.92[b]	−3.20[b]	−2.55[b]	0.001763
Oscillospira	1.77[a]	−3.06[b]	−3.31[b]	−1.69[b]	0.025503
Ruminococcus	4.73[a]	−4.32[b]	−5.51[b]	−4.12[b]	0.000510
Megasphaera	0.06[a]	−3.74[b]	−5.59[b]	−3.12[b]	0.003442
Negativicoccus	1.10	2.07	−0.97	2.83	0.522025
Phascolarctobacterium	0.96[a]	−2.34[b]	−6.12[c]	−2.64[b]	0.016017
Veillonella	0.02	2.05	−2.12	0.09	0.765288
Atopobium	0.02[a]	−2.50[bc]	−4.03[c]	−1.75[b]	0.026410
Collinsella	0.01	−1.07	−1.82	0.15	0.530060
Eggerthella	0.06	−1.13	−0.18	0.78	0.983175
Coprobacillus	0.25	0.01	−0.05	1.69	0.672057
Bulleidia	1.01[a]	−1.83[b]	−3.47[b]	−1.66[b]	0.031552
Eubacterium	0.49[a]	−1.95[b]	−1.51[b]	−2.18[b]	0.011357
Fusobacterium	8.12[a]	2.09[b]	1.91[b]	1.99[b]	0.009146
Sutterella	0.26	−0.39	−2.37	−1.56	0.237870
Bilophila	0.03[a]	−3.26[b]	−2.21[b]	−2.15[b]	0.008941
Desulfovibrio	0.63[a]	−6.10[b]	−5.92[b]	−5.81[b]	0.000004
Escherichia	4.39	1.99	1.24	1.15	0.240319
Aggregatibacter	0.06	3.45	3.87	5.91	0.475903
Pseudomonas	0.05	−1.34	−0.65	−1.47	0.091075
Methanobrevibacter;s__smithii	0.01[a]	−2.50[b]	−3.03[b]	−2.56[b]	0.001626
Bacteroides;s__acidifaciens	0.04[a]	−2.62[b]	−3.95[b]	−3.48[b]	0.004352
Bacteroides;s__heparinolyticus	0.23[a]	−2.92[b]	−3.23[b]	−2.27[b]	0.004990
Bacteroides;s__ovatus	0.37[a]	−2.58[b]	−4.41[b]	−3.45[b]	0.006093

Table 3 (continued)

#OTU ID	% R.Q	Log_2(F/C)			ANOVA*
	Baseline	amox02	amox07	amox08	*p* value
Bacteroides;s__pyogenes	4.18[a]	1.00[b]	1.09[b]	− 1.72[c]	0.025060
Bacteroides;s__uniformis	0.20[a]	− 1.71[b]	− 2.38[b]	− 4.30[c]	0.031170
Bacteroides;s__vulgatus	1.26[a]	− 2.37[b]	− 2.46[b]	− 1.61[b]	0.014477
Parabacteroides;s__distasonis	1.12[a]	− 3.05[b]	− 3.07[b]	− 4.17[b]	0.002280
Prevotella;s__	0.64[a]	− 6.11[b]	− 5.93[b]	− 6.46[b]	0.000009
Enterococcus;s__durans	0.13[b]	4.69[a]	5.14[a]	5.13[a]	0.038408
Enterococcus;s__faecalis	0.03	6.84	7.18	8.13	0.220472
Lactobacillus;s__antri	8.33[a]	− 1.20[b]	− 1.68[b]	− 1.15[b]	0.021077
Lactobacillus;s__crispatus	9.75[q]	− 2.13[b]	− 5.85[c]	− 3.19[bc]	0.018745
Lactobacillus;s__delbrueckii	1.50[a]	− 2.07[b]	− 5.76[c]	− 3.18[bc]	0.020505
Lactobacillus;s__gasseri	9.54[a]	− 2.57[b]	− 4.74[b]	− 2.75[b]	0.008456
Streptococcus;s__hyointestinalis	0.33[a]	− 2.73[b]	− 3.1 [b]	− 2.43[b]	0.002672
Clostridiaceae;Other;Other	0.14[a]	− 3.34[b]	− 2.12[b]	− 2.12[b]	0.011455
Clostridium;s__baratii	0.02	− 0.32	− 0.48	− 0.38	0.054278
Clostridium;s__butyricum	0.01	11.77	13.60	11.35	0.427891
Clostridium;s__cadaveris	0.01	2.85	1.56	4.72	0.059228
Clostridium;s__frigidicarnis	0.01[a]	− 3.63[b]	− 3.65[b]	− 2.69[b]	0.003019
Clostridium;s__perfringens	1.90	− 1.49	− 2.57	− 0.44	0.226359
Finegoldia;s__magna	0.02	4.35	6.71	6.76	0.313308
Dorea;s__	1.01[a]	− 3.11[b]	− 4.97[b]	− 3.22[b]	0.003398
Roseburia;s__faecis	0.02[a]	− 1.17[b]	− 0.70[ab]	− 1.24[b]	0.001921
Ruminococcus;s__	3.12[a]	− 3.28[b]	− 4.35[b]	− 3.14[b]	0.021094
Ruminococcus;s__gnavus	2.47[a]	− 1.76[b]	− 2.69[b]	− 1.58[b]	0.047608
Faecalibacterium;s__	0.45[a]	− 3.12[b]	− 3.35[b]	− 2.67[b]	0.029017
Faecalibacterium;s__prausnitzii	0.02[a]	− 0.91[ab]	− 1.44[b]	− 1.10[b]	0.025503
Negativicoccus;s__succinicivorans	1.10[b]	2.07[a]	− 0.97[c]	2.83[a]	0.015768
Veillonella;other	0.02[c]	4.24[a]	0.30[c]	1.64[b]	0.018016
Adlercreutzia;s__	0.17[b]	− 0.53[b]	1.38[a]	1.17[a]	0.026410
Eggerthella;s__lenta	0.06	− 1.13	− 0.18	0.78	0.135460
Coprobacillus;s__cateniformis	0.25[b]	0.02[b]	− 0.03[b]	1.69[a]	0.031552
Bulleidia;s__	1.01[a]	− 1.83[b]	− 3.47[c]	− 1.66[b]	0.005019
Fusobacterium;s__gonidiaformans	1.45[b]	4.54[a]	4.36[a]	4.45[a]	0.000031
Sutterella;s__parvirubra	0.02[c]	3.44[a]	1.41[b]	2.20[ab]	0.008941
Escherichia;other	4.23	1.99	1.25	1.14	0.266914
Escherichia;s__	0.04	1.74	1.20	0.78	0.222009
Escherichia;s__albertii	0.11	1.98	1.17	1.29	0.475903
Actinobacillus;s__porcinus	0.02[a]	− 3.05[b]	− 4.70[b]	− 4.69[b]	0.003434
Acinetobacter;s__lwoffii	0.19[a]	− 6.22[c]	− 6.28[c]	− 1.93[b]	0.031993

*One-way ANOVA with $p < 0.05$. *R.Q.*, relative quantity. [abc]Letters indicate significant differences within a line by Tukey's honestly significant differences (HSD) test ($p < 0.05$)

after fermentation and approximately 6 times more than amox07. The *Clostridium* group I was significantly reduced at the EP just by amox08 (− 2.32-folds) and significantly increased with amox02 and amox07, of 1.31- and 1.43-folds, respectively. The *Clostridium* group IV was reduced by each treatment and significantly just by amox08, but the reduction scored by amox08 was almost thrice that of amox02. In the taxon *Bacteroidetes*, the BPP group quantified mainly the *Bacteroides* abundance, and recorded significant shifts in reduction for any milk sample, with amox08 having more than the double the strength of amox02. Considering the *Enterobacteriaceae* and the *E. coli* taxa, significant reductions from the BL on were observed just for the amox08 sample at the EP. Similarly, the *Bifidobacteriaceae* were significantly reduced just by amox08, but values under the detection limit were observed for amox07. In conclusion,

Table 4 Enumeration (cells/mL) by qPCR of core microbiota of milk and fermentation samples

Sample	Cells/mL			Log_2(F/C)		MANOVA
	Milk ± SD*	BL raw**	BL mean ± SD	T1	EP	
Eubacteria						
amox02	1.80E + 06 ± 1.50E + 06^{A}	1.26E + 10	1.05E + 10 ± 1.92E + 09^{b}	1.08^{aA}	0.26^{ab}	0.014929
amox07	1.23E + 06 ± 1.12E + 06^{A}	1.01E + 10	1.05E + 10 ± 1.92E + 09	0.54^{AB}	0.31	0.060255
amox08	3.25E + 05 ± 1.21E + 05^{B}	8.84E + 09	1.05E + 10 ± 1.92E + 09	0.19^{B}	0.26	0.822842
Blank	n.a	1.06E + 10	1.05E + 10 ± 1.92E + 09	− 0.43^{B}	− 0.43	0.088726
	0.046181	0.072272		0.034454	0.987142	*p* value
Firmicutes						
amox02	4.16E + 05 ± 2.76E + 05	1.62E + 09	2.73E + 09 ± 9.73E + 08^{b}	0.72^{abA}	1.20^{aaA}	0.037431
amox07	3.37E + 05 ± 1.89E + 05	3.17E + 09	2.73E + 09 ± 9.73E + 08^{b}	1.28^{aA}	1.73^{aA}	0.005016
amox08	7.98E + 04 ± 3.47E + 04	3.41E + 09	2.73E + 09 ± 9.73E + 08^{a}	− 0.29^{aB}	− 4.15^{bC}	0.018042
Blank	n.a	2.92E + 09	2.73E + 09 ± 9.73E + 08	− 0.43^{B}	0.29^{B}	0.276141
	0.061105	0.076691		0.012030	0.000066	*p* value
Bacteroidetes						
amox02	n.a	1.37E + 09	1.68E + 09 ± 3.73E + 08	− 0.85^{A}	− 0.16^{A}	0.089309
amox07	n.a	1.58E + 09	1.68E + 09 ± 3.73E + 08^{a}	− 1.16^{bB}	− 1.44^{bB}	0.002010
amox08	n.a	2.10E + 09	1.68E + 09 ± 3.73E + 08^{a}	− 2.81^{bB}	− 2.67^{bC}	0.001914
Blank	n.a	1.58E + 09	1.68E + 09 ± 3.73E + 08^{a}	− 0.43^{aA}	− 1.28^{bB}	0.001463
		0.970638		0.003979	0.002111	*p* value
Lactobacillales						
amox02	2.97E + 05 ± 2.75E + 05^{A}	1.37E + 09	9.26E + 08 ± 4.18E + 07	0.08^{A}	0.33^{A}	0.064451
amox07	2.53E + 05 ± 1.67E + 05^{A}	1.58E + 09	9.26E + 08 ± 4.18E + 07	− 0.10^{A}	− 0.23^{A}	0.085205
amox08	5.33E + 04 ± 2.89E + 04^{B}	2.10E + 09	9.26E + 08 ± 4.18E + 07 ^{a}	− 2.59^{bB}	− 2.96^{bB}	0.000001
Blank	n.a	1.58E + 09	9.26E + 08 ± 4.18E + 07	− 0.42^{A}	− 0.34^{A}	0.060350
	0.043189	0.999926		0.000002	0.000007	*p* value
Bacteroides–Prevotella–Porphyromonas						
amox02	n.a	5.91E + 08	5.69E + 08 ± 3.23E + 07	− 0.38^{A}	− 0.68^{A}	0.060603
amox07	n.a	5.42E + 08	5.69E + 08 ± 3.23E + 07^{a}	− 1.26^{bA}	− 1.47^{bB}	0.000001
amox08	n.a	6.02E + 08	5.69E + 08 ± 3.23E + 07^{a}	− 2.27^{bB}	− 3.19^{cC}	0.000002
Blank	n.a	5.42E + 08	5.69E + 08 ± 3.23E + 07^{a}	− 0.41^{aA}	− 0.81^{bA}	0.000187
		0.901999		0.000615	0.000569	*p* value
Bifidobacteriaceae						
amox02	1.24E + 04 ± 2.67E + 02^{B}	3.81E + 02	2.35E + 02 ± 1.42E + 02	0.54	0.93^{A}	0.712690
amox07	2.34E + 04 ± 3.63E + 03^{A}	9.68E + 01	2.35E + 02 ± 1.42E + 02	n.d	n.d	n.d
amox08	n.d	2.32E + 02	2.35E + 02 ± 1.42E + 02^{a}	− 4.37^{c}	− 2.65^{bB}	0.039056
Blank	n.a	2.32E + 02	2.35E + 02 ± 1.42E + 02	− 0.15	− 0.13^{A}	0.946939
	0.000001	0.951484		0.088766	0.036499	*p* value
Enterobacteriaceae						
amox02	1.80E + 04 ± 5.36E + 02^{B}	1.30E + 07	1.39E + 07 ± 1.31E + 06	0.68^{A}	1.28^{AB}	0.301428
amox07	2.07E + 04 ± 5.42E + 03^{AB}	1.25E + 07	1.39E + 07 ± 1.31E + 06^{b}	0.96^{aA}	2.56^{aA}	0.000256
amox08	3.50E + 03 ± 2.39E + 03^{A}	1.51E + 07	1.39E + 07 ± 1.31E + 06^{a}	− 1.42^{bB}	− 2.54^{bC}	0.003090
Blank	n.a	1.50E + 07	1.39E + 07 ± 1.31E + 06	0.12^{A}	0.35^{B}	0.073044
	0.000001	0.907242		0.001772	0.000737	*p* value
Clostridium group IV						
amox02	5.07E + 04 ± 9.35E + 03	2.51E + 08	2.51E + 08 ± 1.01E + 07	− 0.55	− 0.57^{A}	0.074610
amox07	1.22E + 04 ± 3.68E + 03	2.45E + 08	2.51E + 08 ± 1.01E + 07	− 0.27	− 0.30^{A}	0.436952
amox08	1.50E + 04 ± 1.17E + 04	2.64E + 08	2.51E + 08 ± 1.01E + 07^{a}	− 1.43^{b}	− 3.02^{cB}	0.000072
Blank	n.a	2.44E + 08	2.51E + 08 ± 1.01E + 07	− 0.42	− 0.27^{A}	0.080431
	0.307957	0.993674		0.079424	0.000007	*p* value

Table 4 (continued)

Sample	Cells/mL			Log_2(F/C)		MANOVA
	Milk ± SD*	BL raw**	BL mean ± SD	T1	EP	
Clostridium group I						
amox02	2.07E + 04 ± 6.10E + 03^{A}	8.02E + 06	1.07E + 06 ± 5.67E + 06	1.05^{A}	1.31^{A}	0.003502
amox07	3.42E + 04 ± 3.15E + 03^{A}	7.03E + 06	1.07E + 06 ± 5.67E + 06	1.21^{A}	1.43^{A}	0.004502
amox08	2.49E + 03 ± 1.16E + 03^{B}	1.73E + 07	1.07E + 06 ± 5.67E + 06	− 1.30^{B}	− 2.32^{C}	0.046666
Blank	n.a	1.20E + 07	1.07E + 06 ± 5.67E + 06	− 0.20^{A}	− 0.28^{B}	0.705529
	0.000071	0.987058		0.000275	0.000003	*p* value
Escherichia coli						
amox02	2.03E + 02 ± 5.33E + 01^{A}	1.50E + 06	1.63E + 06 ± 1.36E + 05^{b}	0.51^{abA}	1.48^{aA}	0.000010
amox07	1.33E + 02 ± 2.64E + 01^{B}	1.58E + 06	1.63E + 06 ± 1.36E + 05^{b}	1.10^{aA}	1.07^{aA}	0.041208
amox08	n.d	1.76E + 06	1.63E + 06 ± 1.36E + 05^{a}	− 1.49^{bB}	− 3.50^{cB}	0.000004
Blank	n.a	1.66E + 06	1.63E + 06 ± 1.36E + 05	0.15^{A}	0.38^{A}	0.064937
	0.000002	0.933157		0.000001	0.000244	*p* value

^{a,b,c}Different lowercase letters on the superscript of values indicate significance difference due to "time category" of MANOVA among a row by Tukey post hoc test ($p < 0.05$). ^{A,B,C}Different uppercase letters on the superscript of values indicate significance difference due to "substrate category" of MANOVA among a column by Tukey post hoc test ($p < 0.05$); *n.a.*, not analyzed; *n.d.*, 0; *BL*, baseline of colonic fermentation; *SD*, standard deviation; *T1*, 18 h; *EP*, 24 h

just amox08 fermentation was able to contain and reduce opportunistic bacteria in piglets' colon, but also reduced the abundance of commensals and beneficials.

Metabolomics

Discrimination of the volatilome of different samples

Through SPME GC–MS, among 18 duplicated cases ($n = 36$), 158 molecules were identified with more than 80% of similarity with NIST 11 MSMS library and the NIST MS Search program 2.0 (NIST, Gaithersburg, MD, USA). On average, 96 were relatively quantified at the BL, while 137 were quantified during the 24 h of experiments at different timepoints. For a landscape description of the volatilome, a dataset of 56 significant molecules (ANOVA $p < 0.05$) was generated, then sorted and super-normalized by similar chemical classes of VOCs, i.e., aldehydes, alcohols, acids and ketones, and other aromatics (alkanes were excluded) (Nissen et al. 2020). In details, within the 17 aldehydes quantified, 6 were found at the BL, 16, 17, and 16 were found during fermentation of amox02, amox07, and amox08, respectively. Within the 14 alcohols quantified, 9 were found at the BL, 13, 14, and 12 were found during fermentation of amox02, amox07, and amox08, respectively. Within the 6 organic acids quantified, 3 were found at the BL, 5, 5, and 3 were found during fermentation of amox02, amox07, and amox08, respectively. Within the 6 ketones quantified, 4 were found at the BL, 6, 5, and 4 were found during fermentation of amox02, amox07, and amox08, respectively. From each dataset, multivariate analyses, such as untargeted principal component analysis (PCA) (Fig. 1) and targeted MANOVA ($p < 0.01$) (Supplemental Table S3 and S4) were achieved to address the specific contributes to VOCs production by the independent variables. Super-normalization of the dataset was essential to unveil the effect of those compounds that are less volatile than others and could be underrepresented, as well as to avoid comparing one chemical class to another.

A PCA of 14 statistically significant alcohols has distributed cases on the plot, discriminating the BL (M11 BL) variables to the fermentation of any milk samples, but not to that of the BC (Fig. 1A). From our results, the main descriptors of fermentation with the milk samples were 1-butanol for amox02, 1-heptanol and 2-nonen-1-ol, (*E*) for amox07, and 1-nonen-3-ol and 2-cyclohexen-1-ol for amox08 (MANOVA $p < 0.01$). Considering the effect of time on the production of these VOCs, the major contributions were derived from the EPs (MANOVA $p < 0.01$).

A PCA of 16 statistically significant aldehydes showed distributed cases on the plot, separating the BL from the fermentations of milk samples, but not from the blank control (Fig. 1B). From our results, the main descriptors of fermentation with the milk samples were 2-butenal, 2-methyl and butanal, 3-methyl for amox02, butanal for amox07, and hexadecanal and 2-nonenal, (*Z*) for amox08 (MANOVA $p < 0.01$). Considering the effect of time on the production of these VOCs, the major contribution for 2-nonenal, (*Z*) was derived from T1 (18 h) and for the others the major contribution was derived from the EPs. (MANOVA $p < 0.01$).

A PCA of 12 statistically significant ketones and organic acids distributed cases on the plot, separating the

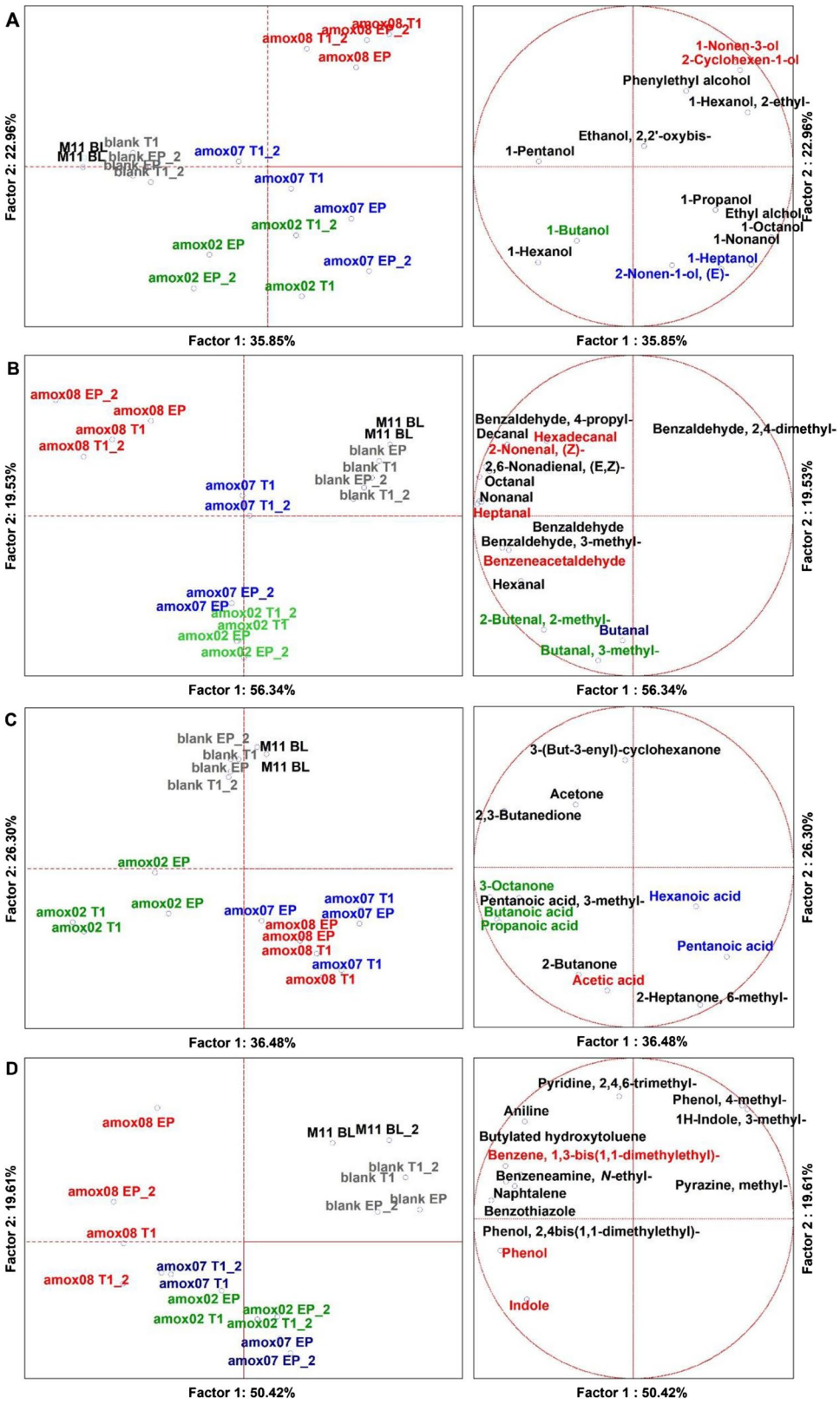

Fig. 1 PCA of the volatilome of colonic fermentation samples. **A** alcohols; **B** aldehydes; **C** organic acids and ketones; **D** aromatic compounds. M11 BL, baseline (2.10 h); T1, intermediate time point (18 h); EP, endpoint (24 h). Different colors on variables indicates respective descriptors by MANOVA ($p < 0.05$) (Supplemental Table S3 and S4)

substrates from each other and from the BL, except for the blank control (Fig. 1C). Descriptors of fermentation were butanoic, propanoic acids, and 2-octanone for amox02, pentanoic and hexanoic acids for amox07, and acetic acid for amox08.

A PCA of 13 statistically significant aromatic VOCs distributed cases on the plot, separating the substrates from each other and from the BL, but not form the BC (Fig. 1D). Otherwise, considering the MANOVA, the main descriptors of fermentation were mainly addressed to amox08 cases. In particular, principal descriptors of this sample fermentation were indole and phenol.

So far, the volatilome of colonic fermentation of mother's milk containing antibiotic residues was described by positive features, such as higher acetic acid, but also by negative ones, such as the higher indole and phenol loads.

Shift of beneficial or detrimental microbial metabolic indicators

To analyze the production of principal volatile microbial metabolites related to food fermentations, we have considered the quantity differences from the BL to the EP, including T1 of eight selected VOCs (ANOVA $p < 0.05$) with renowned bioactivity in humans (short and medium chain organic acids and aromatic compounds). In this elaboration of results, we chose not to include the case of the blank control, because the output generated by volatilome analyses found no discrimination for this case. The first group of VOCs is relative to low organic acids, such as acetic, propanoic, and butanoic acid, that are beneficial compounds essential for the piglets' gut mucosa and the eubiosis of the colon microbiota (Fig. 2A). The second set is relative

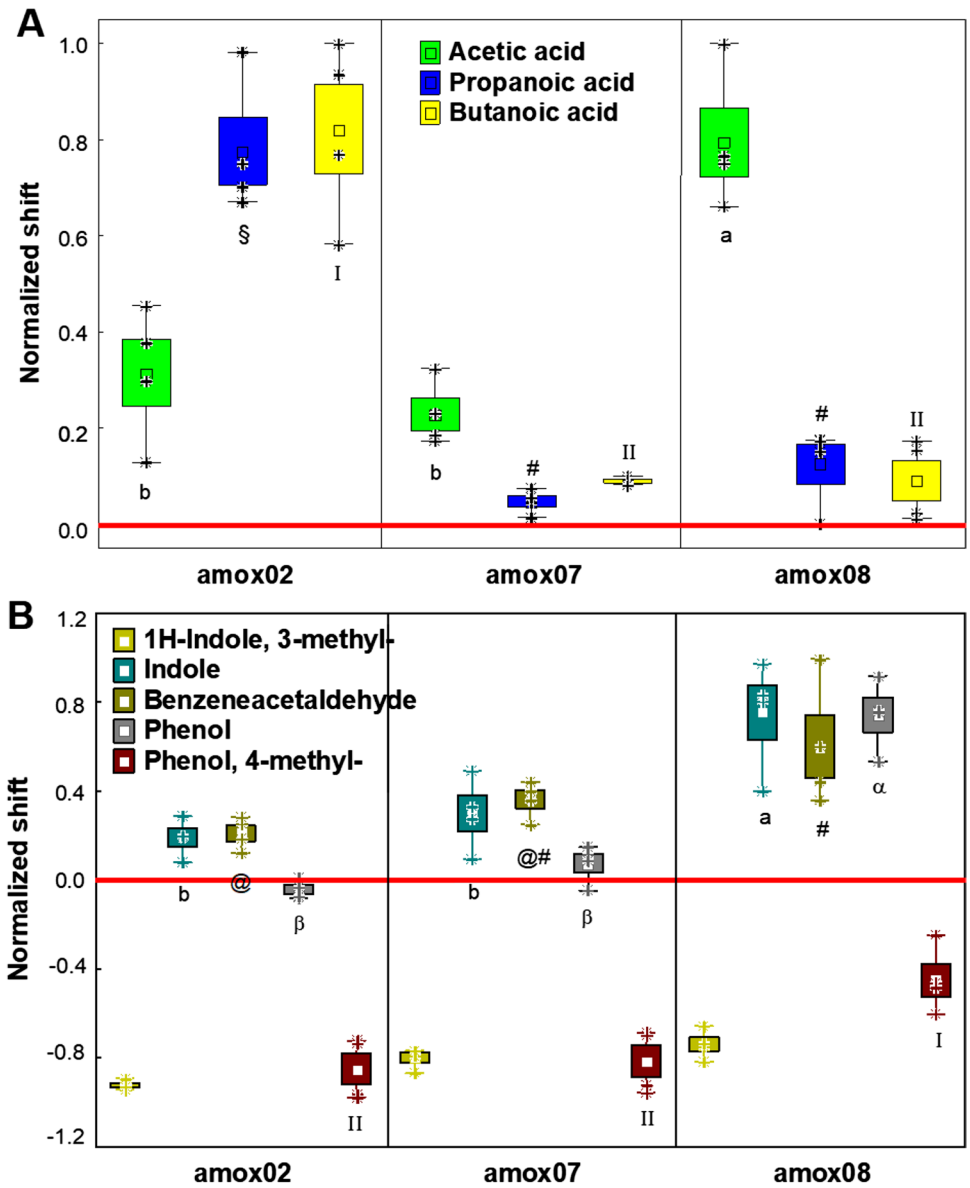

Fig. 2 Changes in the abundance of **A** beneficial microbial VOCs metabolites and of **B** detrimental VOCs metabolites, expressed as normalized scale from relative abundances with respect to the baseline (red line). The baseline absolute quantifications in mg/kg are found in the Supplementary Material (Supplemental Table S2). Changes were recorded after 18, and 24 h of in vitro fecal batch fermentations with amox02, amox07, amox08, and a blank control. Each plot is made with the raw data obtained from each time point and replica. Samples were analyzed in duplicate from two independent experiments ($n = 4$). Marker, mean; box, mean ± S.D.; whiskers, min–max; asterisks, raw data. Cases with different letters or numbers or symbols among a single independent variable are significantly different according to Tukey's HSD test ($p < 0.05$)

to compounds related to proteolytic fermentation and/or detrimental for the piglet's gut mucosa, such as indole, 1H-indole, 3-methyl (skatole), phenol, phenol, 4-methyl (*p*-cresol), and benzeneacetaldehyde (Fig. 2B).

Results shown in Fig. 2A indicate that acetic, propanoic, and butanoic acids concentration was increased from small amounts detected at the BL (Supplemental Table S2), with any milk sample. Specifically, amox02 fermentation produced the top amounts of propanoic and butanoic acid, but little quantity of acetic acid. In contrast, the amox08 fermentation produced the top amount of acetic acid, but little quantity of propanoic and butanoic acid.

Results shown in Fig. 2B indicate that starting from BL values (Supplemental Table S2) detrimental aromatic VOCs concentration trended similarly during any milk sample fermentation. skatole and *p*-cresol were reduced, while indole, phenol, and benzeneacetaldehyde were increased, after colonic fermentation in respect to the BL. In particular, there were significative differences between amox02 and amox08 in the production of indole and benzeneacetaldehyde and in the reduction of *p*-cresol. In particular, the former two were produced 3.9-folds more and 2.8-fold more in amox08 than amox02, respectively.

Discussion

It was reported that early-life in-feed antibiotic treatments could alter the gut microbiota of young piglets, affecting digestive physiology, with greater respect to carbohydrates metabolism (Mu et al. 2017; Lin et al. 2018) and future growth (Yu et al. 2018). Indeed, once ingested, amoxicillin undergoes acid-catalyzed degradation in the stomach and enzymatic degradation by intestinal flora; previous studies showed the presence of various beta-lactamase enzymes in the normal intestinal microbiota of juvenile pigs (Reyns et al. 2008). Being amoxicillin a hydrophilic drug, it can be mainly found in the liquid fraction once ingested through milk (Ozdemir et al. 2018); however, using a gut model adapted to suckling piglets' colon microbiota represents a valuable approach to study gut microbiota shift and their metabolites as a consequence of milk amoxicillin residues absorption.

Microbiomics

Biodiversity of colonic microbiota

From the alpha diversity analyses, the resulting scenario indicates that generally the eubiosis in respect to the BL was maintained after colonic milk fermentations by any index, with the exception for significant reductions in evenness. This effect commonly happens in the in vitro colon models, because underrepresented taxa use to grow slower than the core microbiota, disrupting the evenness of distribution. The Good's index that had no significative differences confirms the stability of MICODE environment throughout fermentation. Beta diversity indicated that the shifts happened in the microbiota of piglets after milk fermentation were not so dramatic and overall the differences among samples from colonic fermentations were limited.

Relative and absolute quantification of colonic microbiota and milk microflora

By the quantifications reported by qPCR analyses, overall, in milk samples the presence of amoxicillin resulted in lower bacterial loads, that desirably were relative to reduction of opportunistic bacteria, but also and undesirably to commensals *Lactobacillales* and to depletion of *Bifidobacteriaceae*. These loads of exogenous microbes should not have impacted on the colon microbiota, because are at least 1,000,000 times lower than what was quantified at the baseline of fermentation.

For the intestinal health of piglets, the capability to reduce the content of opportunistic and pathogens, such as those included in the families *Staphylococcaceae*, *Enterobacteriaceae*, and *Desulfovibrionaceae*, is an important goal, because these bacterial taxa are culprits of dysbiosis induction and can led to intestinal pathogenesis (Gresse et al. 2017; Hasan et al. 2018). For example, the first family is generally transferred to the piglets' colon from the batch flora of the mammary glands and some species are associated with several important piglets' pathologies (Wang et al. 2017).

For the intestinal health of piglets, the *Lactobacillales* order is fundamental. Since the first days, the piglets' colon microbiota is dominated by *Lactobacillaceae* mainly, accounting for a third of the whole pie (Petri et al. 2010). This taxon is inherited from the sow milk, that in our sow milk samples had a mean value of 2.01E + 05 cells/mL, and establish an important symbiosis up to the adult phase, contributing to the microbiota's beneficial effects (Petri et al. 2010). Additionally, several lactobacilli strains of pig's origin were proposed as probiotic and porcine feed additive, e.g., *Lactobacillus salivarius* LS6 (Yeo et al. 2016). However, in our work, this community was reduced by the action of antibiotic residue in milk.

The inhibitory activity against commensals and beneficial taxa is clearly a side effect of wide range antibiotics, such as amoxicillin, that other than the opportunistic taxa also reduce largely the richness of the microbiota, including the split in beneficial bacteria.

For the intestinal health of piglets, the role of *Clostridiales* is crucial, because represents a large portion of the core microbiota, accounting generally for the 30% of the colon

microbiota of piglets. The reduction of *C. perfringens*, *C. baratii*, and *C. frigidicarnis* is fine since are causative agents of enteritis in pigs and used to spread in herds, additionally toxigenic *C. perfringens* can lead to death, and also represent a risk for the consumers (Mehdizadeh Gohari et al. 2021). Also, the increased abundance of *C. butyricum* has to be observed as a positive feature, since this taxon is a butyrate producer and has been proposed and successfully tested as a probiotic for weanling pigs feed (Peeters et al. 2019; Casas et al. 2020).

Considering the overall scenario, there is evidence that some amoxicillin resistant taxa took advantage of the depletion of abundant opportunistic sensitive ones. For example, three are the cases encountered in our work: (i) the split in the *Lactobacillales* class, where the *Enterococcaceae* took advantage from the depletion of *Lactobacillaceae*. Some species of *Enterococcaceae* have been recently used as probiotics for post-weaning pigs (Sato et al. 2019). In contrast, in poultry some species can cause bacteremia, especially during antibiotic treatment, because are reported to be resistant to amoxicillin (Cuccato et al. 2021). (ii) The split in the *Clostridiales* class, which deepened at the lowest taxonomic level, was driven by amox08 fermentation. This taxon was reduced for its overall content, but the reduction was higher for the portion of the more sensitive taxa than that of the tougher taxa. Among these former taxa, there are also some reported to be generally resistant to antibiotic and also specifically to amoxicillin as follows: *Peptostreptococcaceae* and the *Clostridiaceae* (de Jong et al. 2014). (iii) The split in *Gammaproteobacteria* order is described by the constrained growth of *Enterobacteriaceae* and the rise in the abundance of *Pasteurellaceae*. Even in this situation, the reduction of *Enterobacteriaceae* from our results is a positive feature to maintain a healthy colon of the animals, but *Pasteurellaceae* are important pathogens affecting the respiratory tract of pigs, which in the past were susceptible to antibiotic treatments (de Jong et al. 2014), but nowadays are becoming resistant developing specific phenotypes (Gao et al. 2021).

Metabolomics

Volatilome

The results that we have presented have highlighted that in respect to the BL, there were no fermentation differences for the BC, but there were discriminations in respect to the milk fermentations, and that each one had typical descriptors mainly produced at the EP. This means that the fermentations of milk substrates produced different profiles of VOCs, because made a different impact on the colon microbiota.

For example, among alcohols the production of 1-butanol described the milk with no antibiotics, while 2-cyclohexen-1-ol that of amox08. The colon microbiota produces different alcohols during fermentation of dietary polysaccharides. For example, 1-butanol is a product of butanoic acid fermentation that happens when the pH is not low enough to ensure the exclusive activity of lactic acid bacteria, as should happen in a healthy piglet colon, maybe due to the action of clostridia. In fact, it is reported that *Clostridium acetobutylicum* produces less acids and more neutral products like butanol, thus carrying out acetone butanol fermentation (Ciani et al. 2013). 2-Cyclohexen-1-ol was probably produced from microbial transformation of amoxicillin building blocks, like cyclohexenone (Jiang et al. 2020).

Among aldehydes, benzeneacetaldheyde and 2-butenal, 2-methyl were found to be descriptors of fermentation of milks with and without antibiotic residues, respectively. The aldehydes that are a result of microbial fermentation of lipids could be health-promoters, like 2-butenal, 2-methyl that was reported to limit the growth of several intestinal pathogens at a very low concentration (Zhang et al. 2020) and could have contributed to the management of a natural occurring eubiosis of colon microbiota. Also, other aldehydes are detrimental, being cytotoxic at a low threshold, such as benzeneacetaldheyde (Zhang et al. 2020), that in our work could have been derived from bacterial fermentation of phenylalanine, that is typically rich in milk proteins. The higher amount of this aldehyde found after fermentation of milk with antibiotic residues could have been produced by the higher abundance of *E. faecalis* that characterized the end of amox08 fermentation. In fact, this taxon is known for its selectivity in fermentation of phenylalanine in respect to lactobacilli (Canon et al. 2021).

Other descriptors of the volatilome that discriminated the fermentation with and without antibiotic residues were the organic acids, with acetic acid for the amox08 and butanoic and propanoic acids for amox02, and also indoles that described principally amox08. These compounds will be discussed later.

Dysbiosis metabolite indicators

A reduction in acetic, propanoic, and butanoic acids abundances is linked to dysbiosis of the colon microbiota and a reduced intestinal cell homeostasis (Gibson et al. 2017). Thus, from our results, no sample was able to disrupt the proper colonic fermentation of milk, because the three of them increased the production of these VOCs in respect to the BL. The different scenario observed in the production of low organic acids could be principally addressed to the increased abundance of enterococci to the reduction of lactobacilli for the production of acetic acid, and to the reduction of butyrate producers bacteria (e.g., *Ruminococcaceae* and *Lachnospiraceae*) seen in amox08.

Enterococci have pyruvate dissimilation that follows several pathways leading to at least five fermentation

end-products including acetate (Snoep et al. 1991). In line with our results, Fujita et al. (2020) reported that the supplementation of pigs fed with *Enterococcus faecium* increased fecal acetate levels, which plays an important role for maintaining immune functions. Oppositely, the reduced microbial production of butanoic acid seen in amox08 in respect to amox02 has to be linked to the undesired inhibitory effect of the antibiotic residues towards sensible commensal *Clostridiales* that are butyrate producers. In particular, we have observed a reduction in *Roseburia* and *F. prausnitzii*. Butanoic acid in piglets is produced mainly by the colon microbiota and is a preferential nutrient for energy production by the colonocyte (Kien et al. 2002). Also, it is an important modulator of gut cellular homeostasis, and when it is administered in diet as sodium butyrate alleviates diarrhea symptoms and decreased intestinal permeability without affecting the growth of early weaned piglets (Feng et al. 2018).

In pigs, skatole and indole are formed by the microbial degradation of L-tryptophan in the large intestine and contribute to the typical development of boar taint (Witte et al. 2021). L-Tryptophan accumulates especially in the colon when protein sources are used with a low pre-cecal digestibility (Leong et al. 2011).

The reduction of these compounds is due to the liver, but when their concentrations is excessive can accumulate also in the adipose tissue (Witte et al. 2021), resulting in a commercial loss. From our results, the higher increase in indole of amox08 in respect to amox02 could be due to the reduced abundance of *Lactobacillaceae* observed in the presence of antibiotic residues. In fact, this taxon in the colon can transform indole into beneficial compounds (e.g., indole propionic acid) (Konopelski and Mogilnicka 2022).

Similarly to indoles, phenol and *p*-cresol are derived from proteolytic fermentation of undigested or partially digested proteins and have been shown to damage the gut mucosa disrupting the epithelial barrier function and being genotoxic (Al Hinai et al. 2019; Wang et al. 2020). Also in farm animals, the excessive production of these metabolites can affect the quality of meat and milk and is a source of contaminating emissions from animal manure (Gasaly and Gotteland 2022). In our work, phenol and *p*-cresol should be derived from fermentation of tyrosine due to proteolysis of milk. From our results, the capacity of amox08 fermentation to reduce less the amount of *p*-cresol than what the control milk did could still be attributed to a lower content in *Lactoabacillaceae*, as it has been reported in a similar in vitro colon model, where the correlation among lactobacilli and *p*-cresol was negative (Al Hinai et al. 2019).

Swine model

Within antibiotics for use in animals, the European Medicines Agency (EMA 2020) has currently classed amoxicillin, without beta-lactamase inhibitors, as category D antibiotic; therefore, it is highly recommended as first line treatment and, as always, should be used prudently only when medically needed (EMA/CVMP/CHMP/682198/2017). The establishment of the intestinal microbiota is a pivotal step in newborn piglets; thus, the effects of antibiotics such amoxicillin in early-life stages could critically affect gut microbial development and future growth (Mu et al. 2017; Lin et al. 2018; Yu et al. 2018). This statement is especially true amid zootechnical industry, where intensive farming pigs undergo fast and massive weight increment. The present in vitro experiment using an innovative colon model allowed authors to carry out a preliminary study avoiding any unnecessary harm to the piglets and the sows as well, still obtaining reliable data on microbial shift due to amoxicillin residues in sows' milk.

Digestive enzyme secretory patterns seem to be of relevance in the process of assimilation of milk components; indeed, previous studies showed that maturation of gastric, pancreatic, and biliary digestive fluids occurs at an early period of life, starting gradual maturation around the sixth day of life (Manners 1976; Corring et al. 1978; Harada et al. 1988). To the authors' knowledge, there are yet no researches that evaluated amoxicillin digestion and absorption in newborn piglets. In this study, 7 days old piglets' fecal samples to build up the in vitro colon microbiota model were used; therefore, as the newborn had an immature digestive capacity, the milk samples were directly fermented in the colon model with no gastric phase digestion.

In conclusion, the early establishment of a stable gut microbiota is pivotal for the pigs' gastrointestinal physiological functions, also affecting future growth performance and therefore, investigating exogenous molecules effects on these indigenous microbes is of great importance in swine production. In this work, the pig model was adopted to study the role of sow's milk in modifying antibiotic resistant gut microbiota for the first time in combination to a gut model. Moreover, a wider understanding was allowed by a metabolomic approach. The use of MICODE, a robust and versatile in vitro model, together with multivariate statistics visibly demonstrated a suitable approach to describe the effects generated by milk containing amoxicillin residues towards the colon microbiota of suckling piglets. To fully understand the transfer of antibiotic from sow's milk to the piglets, in vivo trials are imperative; however, the results presented are target-effective and should be reliable for preclinical investigations. Due to the results obtained, this experimental approach looks suitable to study some mechanisms of antibiotic resistance transfer as well. Furthermore,

such in vitro approach could be included in a pipeline of experiments reducing the number of living animals testing, according to the Directive 2010/63/EU and the Regulation (EU) 2019/1010.

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Author contribution LN, AG, MLB, and AZ conceived and designed the research. DV, AE, and CA were responsible for the animal trials including samples collection. FC and LN conducted the in vitro experiments. LN and FC analyzed data. LN, FC, CA wrote the manuscript. AG, MLB, DV, AE supervised the work. MLB funded the study. All authors have read and agreed to the published version of the manuscript.

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Data availability Data are available upon reasonable request to the corresponding author. The NCBI Bioproject PRJNA862673 is available at https://www.ncbi.nlm.nih.gov/bioproject/862673, including Biosamples and relative SRAs, that will be release at least 2022–12-15, or with the release of linked data, whichever is first.

Declarations

Ethics approval All applicable international (DIRECTIVE 2010/63/EU), national (Italian Ministry of Health), and institutional (University of Bologna) guidelines for the care and use of animals were followed. Experimental protocol approved by the Local Ethics Committee and the Italian Ministry of Health (Legislative Decree 26/2014, authorization n° 32/2021-PR, protocol number 2216A.17).

Conflict of interest The authors declare no competing interests.

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Conclusions and Future Applications

Selecting the most reliable animal species for lactation experimentation was a difficult process, which frequently implied the necessity to overcome knowledge and information gaps. The theme discussed within this project represents the founding for a conscious selection, where anatomical and physiological resemblance to humans is the principle of a reliable translational value.

1. Preliminary step

By observing the 1. Preliminary step (Ventrella et al. 2021) results, Non-Human Primates (NHPs), as expected, could have represented the prime choice to build a lactation animal model. However, additional factors needed to be considered. In the last decades, the biomedical research community has commonly agreed to use experimental NHPs exclusively if alternatives are unavailable. Indeed, experimentations entailing such species are very expensive, long to organize, and low sample sizes are required due to ethical considerations. Lastly, collecting milk from NHPs would be highly challenging, considering the solid maternal behavior. Difficulty of sample collection should always represent a pivotal element when establishing experimental protocols. Indeed, selecting a "difficult" species might lead to failure, particularly in chronic/long studies requiring un-sedated animals and periodic samplings. Tinier animals such as rodents and rabbits are not certainly the primary choice for lactation trials, considering both the impossibility to obtain harmless and repeated milk/blood samples, and the physiological impairment when compared to human lactation process.

In such scenario, larger species like dogs and pigs were considered to be appropriate for lactation studies. Similarly to NHPs, the enrollment of dogs for experimental purposes raises strong disapproval within society, especially in Europe. On the contrary, the use of pigs in biomedical research appears more widely approved and allowed as a solid alternative option. Amid swine breeds, minipigs provide all the anatomical and physiological similarities to humans while avoiding the major problem of conventional breeds represented by the size. In particular, Göttingen Minipigs are

precisely bred for biomedical research, therefore they usually have a highly standardized genetic background and health status.

The literature analysis performed within the 1. Preliminary step represented a crucial stage of the complex decisional process that brings to the selection of an animal species for *in vivo* lactation trials. In general, the swine species, with emphasis to minipigs, were considered the primary choice relating to both physiological affinity with humans and the realistic feasibility of *in vivo* lactation studies.

2. First step

Once the swine species was chosen, the first trial (2. First step) was designed to assess if this innovative lactation model would have been capable of generating quantitative and translatable data. The experiment was done at the Department of Veterinary Medical Sciences, Alma Mater Studiorum – University of Bologna with both conventional hybrid and Göttingen minipig sows, opening the pathway for future studies. Indeed, this "large" animal model allowed for multiple samplings and pregnant sows were quite easy to train, when the appropriate positive reinforcement training protocol was employed. Main refinements of this experimental procedures were the above-mentioned training and the peripheral central venous catheter that allowed for harmless recurrent blood samplings.

In spite of the demanding logistics that working with conventional animals require, large hybrid sows showed excellent maternal behavior. Additionally, their litter are usually numerous and with bigger piglets compared to minipigs of the same age, thus blood sampling was reasonably easier, allowing for larger volumes as well. However, the research team is undoubtedly aware that such trials with adult conventional sows weighting up to 300 Kg could never be employed amid pharma industry settings. For the latter reason, the veterinary team from University of Bologna decided to enroll farm hybrid sows just for the first trial, to have an actual perception of the overall feasibility of the study, considering both researchers' and animals' perspective, before moving to Göttingen Minipigs. Despite the manpower needed for both trials (conventional and

minipigs) the experimental procedures were substantially feasible and quite well tolerated by the animals. Indeed, sows and piglets did not exhibit severe discomfort-related behavior during the entire length of the study.

Amoxicillin was selected for the first *in vivo* trial for multiple reasons. It is commonly used in porcine medicine as therapeutic molecule; therefore a good amount of pharmacological background data was already reachable. Additionally, another ConcePTION Work Package used amoxicillin in parallel for a clinical study, which will hopefully allow researchers to combine *in vivo*, *in vitro* and clinical trials results.

Upon samples analysis, amoxicillin was always quantifiable within the range of detection in sows' plasma and milk, corroborating the hypothesis that amoxicillin is able to cross the blood/mammary barrier. However, the compound concentrations do not match between the two matrices: amoxicillin peak levels in plasma and milk were registered respectively after 2- and 4-hours post-administration. This result highlighted a notable delay in the secretion of amoxicillin into milk from the blood/mammary barrier. It is authors' opinion that for this reason the molecule was almost never founded in piglets' plasma, since these samples were collected 2.5 hours after maternal administration, while the amoxicillin max concentration in milk was recorded only 4 hours after dosing. When matching milk samples and the corresponding piglets' plasma, the difference suggests that at that specific time point the litter exposure to amoxicillin in milk was yet too low.

Unfortunately background data for milk were unavailable, thus the interpretation of the piglets' plasma results was hard and only inferential. Thanks to the first WP3 *in vivo* trial data on amoxicillin secretion into milk are now available. It is author's opinion that, upon study design adjustment, collecting piglets' blood at 4 hours post maternal administration will most likely provide different results. Therefore for future trials regarding amoxicillin, this additional blood sample for piglets should be added to allow a better knowledge of such molecule transfer into milk and subsequent intake from nursing piglets. On the other hand, for trials involving other molecules, time points of

sampling will be carefully tailored according to the hereby results and the available pharmacokinetics data in the swine species.

Parallel to the development of the *in vivo* model, attempts are ongoing to set up and classify *in vitro* porcine epithelial blood-milk barrier using swine mammary epithelial cells (PMECs) (Bernardini et al. 2021). *In vivo* milk to plasma data enables an in-depth comparison with *in vitro* results obtained from the very same species, increasing trust and reliance in the *in vitro* to *in vivo* extrapolation (IVIVE) that can be used for PK and PD studies translatable to humans. Moreover, the high-resolution results in (mini)pigs with one specific pharmacological compound will be crucial to the refinement of PBPK-based (*in silico*) prediction of drug milk excretion. The merger of these complementary model systems will be pivotal for the advancement of a public platform, which should be meant to be a useful support for future prediction of drug PK during breast feeding. The envisaged platform will fulfill the ConcePTION WP3 objective: proving the best predictive tools and models for study medications transfer into breast milk.

3. Second step

As previously seen, within the Russel and Burch 3Rs principles, Reduction is about minimizing the number of animals used per experiment, remaining consistent with the scientific objectives. It has to be acknowledged that for a faithful reduction of living animals it is crucial to build a trustworthy network within the institute of biomedical research, either public or private, in which multiple experiments occur. Indeed, different research teams usually coexist amid one University or industry and each of them could eventually benefit from the exchange of samples; a useless matrix for someone could represent a valuable source for other sorts of research. It was in such scenario that the ConcePTION WP3 task 3.3 veterinarians teamed up with the microbiologists from the Department of Agro-Food Sciences and Technologies, both research groups belonging to the Alma Mater Studiorum – University of Bologna. Indeed, to obtain as much informations as possible from 2. First Step trial with

amoxicillin, the authors decided to get samples of feces (harmless) from the suckling piglets to study the action of such antibiotic in milk, in regard to newborns' colon microbiota (3. Second step).

Indeed, the development of the physiological gut microbiota is a critical moment in young mammals, thus the consequences of amoxicillin residues assumed through milk in the first weeks of life could significantly interfere with animals' digestive physiology and growth.

Samples obtained from the first ConcePTION WP3 *in vivo* trial allowed the development of an innovative *in vitro* colon model adapted to suckling piglets, representing a valuable methodology to study microbiota shifts due to assumption of antibiotics residues through milk. *In vitro* guts models are a useful resource for this field of research since they focus on the alterations in the core microbial groups and their metabolites, allowing the assessment of their composition and abundance over time, mimicking what happens in *in vivo*. In this study, it was used the Multi-Unit In Vitro Colon Model (MICODE) (Nissen et al. 2021) to simulate the proximal colon of piglets and aiming to extend the knowledge on antibiotic transfer through milk and antibiotic resistance phenomenon in such young animals.

The results exhibit an impactful effect of amoxicillin in newborns microbiota and its metabolites composition. Nevertheless, antibiotic residues did not alter the overall biodiversity: it was highlighted a limitation of pathogens and opportunistic taxa, such as Enterobacteriaceae and commensal Lactobacillaceae, along with a reduction in commensal Ruminococcaceae and beneficial Bifidobacteriaceae. On the other hand, and most important, some amoxicillin resistant species raised, like Enterococcaceae and Clostridiaceae (Nissen et al. 2022).

In conclusion, to the author's knowledge, this multi-stepped study represents the first research evaluating the action of amoxicillin transfer through milk and its effects on piglets' wellbeing and gut microbiota. Even if no adverse effects were encountered in the *in vivo* trial (2. First Step**)**, it appeared clear how antibiotic drugs given to lactating sows could have a wide impact on the development of the complex microbial ecology of the litter (3. Second step) (Nissen et al. 2022).

From a translational point of view, because of the extensive health advantages of breastfeeding, the building of such innovative swine lactation model could represent a pivotal tool to evaluate the exposure and assess the risk to the nursing child when the mother needs medications, either "acute" or "chronic". This experimental approach represents target-effective results that should be reliable for pre-clinical PK investigations, which hopefully in a near future would be systematically performed, allowing biomedical staff to provide more correct information to parents, who deserve to receive the best care for their own health and that of their newborn.

Results of such researches could benefit veterinary medicine as well, especially in the last years, given the ongoing global pandemic of coronavirus disease 2019, scientists from all over the world have been forced to give great importance on integrating human and animal health issues. As Doctor Rudolf Virchow (1821-1902) wisely stated a century ago "Between animal and human medicine there is no dividing line—nor should there be. The object is different but the experience obtained constitutes the basis of all medicine."

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