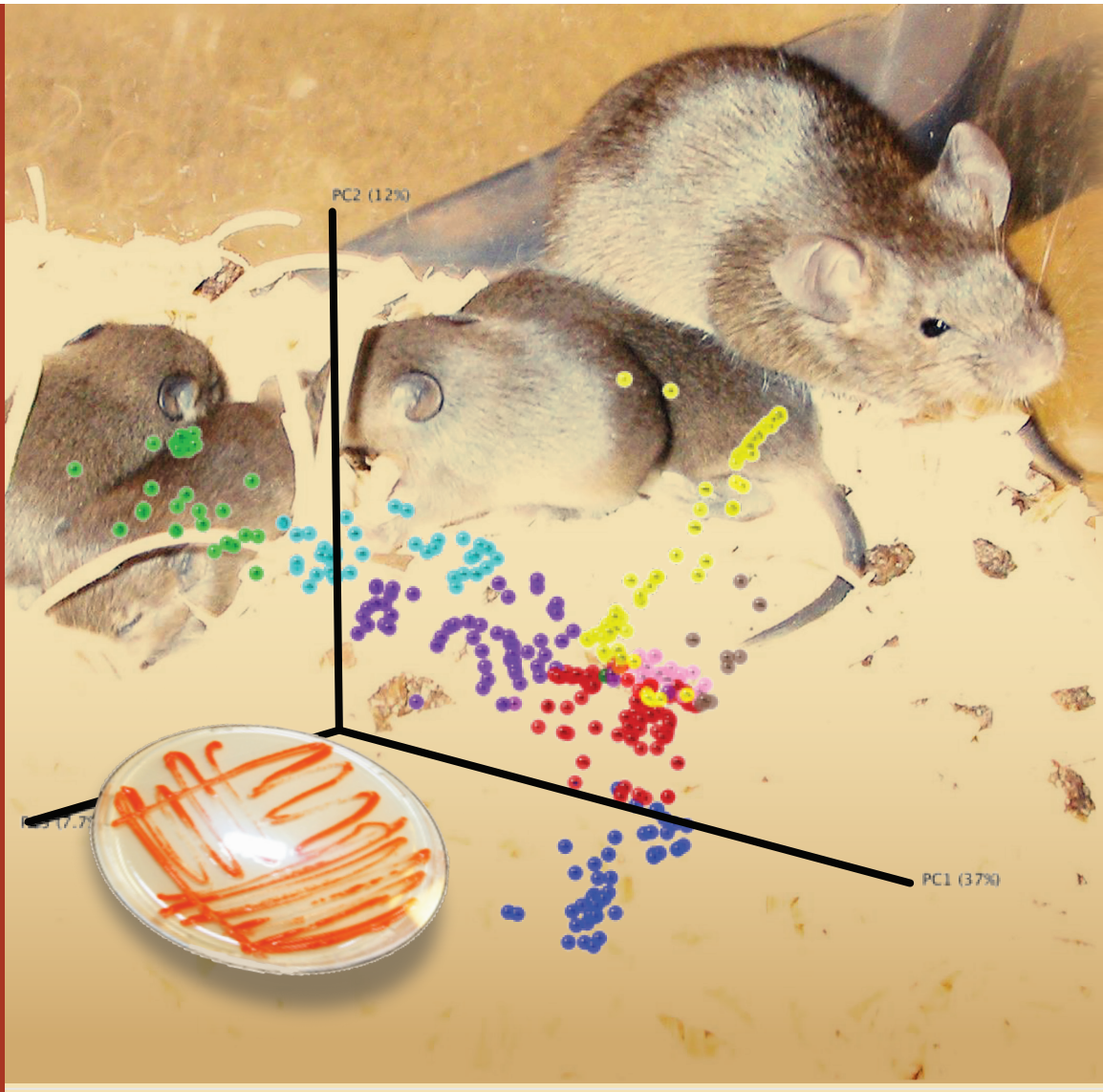

SECOND EDITION

Handbook of Laboratory Animal Bacteriology

Axel Kornerup Hansen
Dennis Sandris Nielsen

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Preface

When the first edition of *Handbook of Laboratory Animal Bacteriology* was published in 2000, it was after a decade during which more formal standards, practices, and guidelines had made bacteriological examination of laboratory animals an important part of securing the quality of these animals for research purposes. At the millennium, there was an increasing common understanding on which agents to look for, while the methods on how to do it differed significantly between laboratories. None of the guidelines released during the 1990s gave any detailed advice on how to perform bacteriological study in laboratory animals. Molecular biology was in its very beginning within bacteriology, and cultivation was the tool applied by all laboratories. The better ones among these would be able to cultivate maybe 15–20% of the bacterial species present in an animal; therefore, those organisms listed in quality assurance programs were those that could be cultivated. Today, molecular biology is in many contexts taking over as *the* tool within bacteriology, and in our labs, molecular biology in some form covers most of what we do. This has shown us that laboratory animals of today harbor a range of so far noncultivable organisms with a much larger impact on animal models than could ever be expected from some of those isolated by simple cultivation techniques. This knowledge, however, is still to be recognized among laboratory animal vendors and health-monitoring laboratories, but we hope that with this book we can broaden the understanding on which bacteria make a difference when modeling human diseases in animals and how we should characterize their presence in the animals.

The first part of the book provides information on the general aspects of bacteriology and how to sample and identify bacteria in the animals; as something new, we have tried to give brief insight into how these bacteria interfere with our animal models and the methods used to investigate this. In the second part of the book, the various bacterial species to be found in laboratory rodents and rabbits are described. While in the first edition this was divided according to cultivation and Gram staining characteristics, which was the standard then, it is now divided according to a more systematic phylum-based approach, which the new molecular

biological methods have made standard. This means that bacterial species previously regarded as closely related now may appear in two different phyla. Also, these chapters cover a range of bacteria identified to be of importance in laboratory rodents and rabbits over the past 10–15 years.

We wish to express our sincere thanks to all those master's and doctoral students, postdoctoral students, and laboratory technicians who over the years have helped us to perform all these procedures. Especially, we are grateful to the two young investigators, Lukasz Krych and Camilla Hartmann Friis Hansen, who in our labs have been the driving forces in describing the impact of bacteria on animal models of human diseases. Last but not least, we want to thank our wives, Inge and Mette, for not being too annoyed over the nights we spent writing this book.

Axel Kornerup Hansen, DVM
Professor
University of Copenhagen

Dennis Sandris Nielsen, PhD, MSc
Associate professor
University of Copenhagen

About the Authors

Axel Kornerup Hansen graduated in 1985 as a doctor of veterinary medicine (DVM) from the Royal Veterinary and Agricultural University in Copenhagen, Denmark. After two years in a small-animal hospital, he became head of laboratories at Møllegaard Breeding Center, today part of Taconic Limited. In 1993, he became associate director at the Department of Experimental Medicine, Faculty of Health Sciences, University of Copenhagen. In 1996, he was awarded the degree of doctor of veterinary science based on his thesis on the impact of bacteria in laboratory rats. Since 1997, he has been a professor in laboratory animal science and welfare at the University of Copenhagen (formerly Royal Veterinary and Agricultural University). In 2000, he was made charter diplomat and together with six others founded the European College of Laboratory Animal Medicine. His research area has primarily been on how the microbiota has an impact on laboratory animal models for inflammatory diseases and how this works in conjunction with the diet. He has published 157 peer-reviewed papers in scientific journals and 36 other works in books, proceedings, or popular journals.

Dennis Sandris Nielsen graduated in 2002 with an MSc in food science from the Royal Veterinary and Agricultural University in Copenhagen, Denmark. During his PhD studies (awarded 2006) and postdoctoral years, he has specialized in microbial ecology, with a special interest in gut microbiota, indigenous African fermented foods, and fermented products contributing to the more joyful side of life (cocoa, wine, coffee). This work was obviously not done alone and involved longer and shorter research stays at Max Rubner Institute, Karlsruhe, Germany; Manchester Metropolitan University, United Kingdom; Université de Abidjan, Cotonou, Benin; Food Research Institute, Accra, Ghana; and Cocoa Research Institute of Ghana, New Tafo, Ghana. In 2010, he was appointed associate professor in the Department of Food Science, University of Copenhagen,

with special responsibilities concerning gut microbiota and probiotics; he now heads a research group mainly focused on microbe–host and microbe–microbe interactions in the mammalian gastrointestinal tract. He has published 69 peer-reviewed papers in scientific journals and more than 15 other works in books, proceedings, or popular journals.

chapter one

Laboratory animal bacteriology

The past, the present, and the future

Contents

1.1 Foundation of the discipline of bacteriology

The French chemist Louis Pasteur, the German biologist Ferdinand Cohn, and the German physician Robert Koch are seen as the three main founders of the discipline of bacteriology in the late nineteenth century. Robert Koch is mostly known for his four "Koch's postulates"; that is, to be regarded as a pathogen, a microorganism must be

1. found in abundance in all organisms suffering from the disease but should not be found in healthy organisms.
2. isolated from a diseased organism and grown in pure culture.
3. a cause of disease when introduced into a healthy organism.
4. reisolated from the inoculated, diseased experimental host and identified as identical to the original specific causative agent.

Koch rather quickly disregarded his first postulate because he realized the existence of latent subclinical infections,[1] which in laboratory animal bacteriology today is also the most common condition for bacterial

infections. The second and the fourth postulate can be questioned as well because many infections (caused by, e.g., *Spirillum minus*, which for years has been known as a potential cause of rat bite fever) cannot be cultivated. What is left today is the third postulate, which still seems to have some validity when discussing primary pathogens, such as *Salmonella*, but also is under heavy pressure because many bacteria act in conjunction with other agents. Additionally modern techniques have been used to show that diseases such as diabetes and colitis not primarily seen as infections are also under strong bacterial impact (e.g., from bacteria such as *Akkermansia muciniphila*, which may act protectively against some diseases[2] and induce other diseases[3]). In general, the postulate works poorly for complex pathogeneses, such as those of cancer viruses.[4] On the other hand, Koch's third postulate should not be fully disregarded; that is, an agent must be proved to make some sort of difference in the host organism if it is to be regarded as causative of a pathophysiological condition.

Robert Koch was also the first one to use a solid medium for bacterial cultivation by adding albumin, sterilized vegetables, or starch pastes to gelatinized meat extracts.[5]

1.2 The need for securing the absence of zoonoses

The rat was the first animal to be domesticated for scientific purposes, and the papers on the use of rats for research increased in the second half of the nineteenth century. The alternative to the use of domesticated rats were wild trapped rats, which all were infested with fleas. This presented a risk of spreading pest bacilli, at that time known as *Bacillus pestis*, later as *Pasteurella pestis*, and today as *Yersinia pestis*, and therefore such rats needed to be decontaminated with hazardous chemicals, such as Pinch's oil gas.[6] Other hazardous zoonoses brought in from the wild were *Leptospira*, *Salmonella*, and *Streptobacillus moniliformis*, for example. These were among the first bacterial infections to be eradicated from laboratory rat colonies, but as late as the 1950s reports appeared on laboratory rats infected with *Leptospira*,[7] while *Streptobacillus moniliformis* was isolated from laboratory rats and mice as recently as the 1990s.[8,9] One important need within laboratory animal bacteriology in the early ages of this discipline was therefore securing the absence of such organisms.

1.3 Eradication of bacterial pathogens

1.3.1 The early age of laboratory animal pathology

In 1913, Theobald Smith at the Harvard Medical School in the Boston area described pneumonia in guinea pigs caused by infection with *Bacillus bronchisepticus* (M'gowan),[10] today known as *Bordetella bronchiseptica,* and

in 1917, Ernest Edvard Tyzzer, also at the Harvard Medical School, visualized that a fatal disease with liver necrosis in mice was caused by a long, slender rod that he named *Bacillus piliformis*.[11] The disease hereafter became known as Tyzzer's disease, and when molecular biology enabled further description of the agent, it was renamed *Clostridium piliforme*.[12] A few other works from that time described bacterial infections in research animals. These were, however, few, and rodents still died from infectious diseases in research facilities for decades, but the pioneer works of Smith and Tyzzer showed that there was by then some understanding that certain infections were hazardous for the animals, leading to high losses and thereby reducing the number of animals available for investigations. For many such diseases, the problems were not solved in a preventive manner, but diagnosed, often simply by morphological pathology, when occurring. Animal welfare did not seem to be the key issue until the 1950s, when there arose an increasing understanding that fewer animals should be used in experiments, and the fact that some animals suffered and died for other-than-experimental reasons, such as infectious diseases, was not acceptable. This culminated with the book, *The Principles of Humane Experimental Technique*, by William Russell and Rex Burch in 1959, which introduced the principles of reduction, refinement, and replacement, known as the 3Rs.[13]

1.3.2 Specific pathogen-free animal breeding and health monitoring

Very much in the new spirit of the 3Rs, Henry Foster, who in 1947 had founded a breeding company in Boston and named it after the Charles River, in the late 1950s reported the use of cesarean section and barrier protection as a way to produce specific-pathogen-free (SPF) rodents on a large scale,[14] which also started a new era of commercial breeders supplying quality animals for research rather than the previous in-house breeding colonies with all their infections and infestations. As "old enemies" were eradicated, new bacteria, such as *Citrobacter rodentium* (by then *C. freundii* type 4280),[15] described by Stephen Barthold from the University of California at Davis, were discovered as causative agents of problematic disease outbreaks, in this case transmissible colonic hyperplasia in mice,[16] and eliminated from the breeding colonies. Another new pathogen was *cilia-associated respiratory (CAR) bacillus,* which was isolated by James Ganaway from the National Institutes of Health in Maryland in 1984; this was described as the cause of chronic respiratory disease in rats, characterized as some sort of *Flavobacterium* or *Flexibacterium*,[17,18] and eliminated from infected colonies before fully systematized.[19] Today, only a few of the bacterial agents commonly observed in the 1950s are actually found during routine screenings of rodent colonies.[20–22]

1.3.3 New agents with a research-interfering potential

Through the 1970s and the 1980s, conventional breeding companies were all outcompeted by breeding companies producing only barrier-protected rodents subjected to a thorough health-monitoring program. The response to finding any agents on the list in the beginning was to report them, but hard competition in the long run closed down this option and made the breeding companies eradicate these unwanted bacteria from their colonies. In the early years, it was very much up to the company to decide what to screen for, but in Europe the first set of common standards was introduced in 1994 by the Federation of European Laboratory Animal Associations.[23] This speeded up the process of pathogen eradication. However, as the more severe pathogens were eliminated, new ones with a more discrete impact on the research models would appear. In the 1990s, James G. Fox from the Massachusetts Institute of Technology isolated and characterized a number of well-defined species of *Helicobacter* from several rodent species. Most of them were not linked to specific pathology, but some (e.g., *H. hepaticus*) seem to be able to cause liver tumors in mice.[24] This illustrates how laboratory animal medicine had now developed from dealing with lethal diseases killing entire colonies to dealing with more discrete diseases interfering with the application of the animals as models for human diseases.

1.3.4 The development of health monitoring

Along with the protective types of animal breeding, health monitoring that is more systematic developed. In the 1960s, bacteriology was seen as more or less synonymous with cultivation, which was the main means of diagnostics for bacteria. Bacteria, such as *C. piliforme* (by then *B. piliformis*), that could not be cultured were screened by pathology of target organs. For viruses, cultivations were not a diagnostic option; therefore, serological screening for antibodies was developed and refined from early methods, such as the complement fixation assay evolved to more modern solid-phase assays such as the enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA), which today seem to be replaced by high-throughput multiplex assays. For some bacteria, this was also the case. In the early 1970s, for instance, it was published that IFA could be used to diagnose *C. piliforme* in infected mice,[25] which through the 1980s became the routine practice in some health-monitoring programs.

This revealed that a large number of European rat colonies apparently seemed to be infected,[26] which gave rise to some debate. On the one side, it called for a new way of thinking among laboratory animal breeders, who had to face that, for some diseases, improvement in production conditions had removed some of the environmental inducers, while the agents were still subclinically present in their colonies. On the other side,

microbiologists had to realize that serology for bacteria carrying a wide range of antibody-inducing epitopes caused far more cross-reactions than viral serology. For an agent such as *C. piliforme*, there seemed to be a true difference between Europe and North America, as many European rat-breeding colonies had been started on the basis of infected reference colonies; furthermore, an agent capable of producing extremely resistant spores would obviously prove difficult to eliminate from the physical surroundings. As the issue of oral tolerance for gut bacteria was never considered (i.e., the fact that the host develops a tolerance toward gut bacteria colonizing in early life preventing, among other things, the formation of systemic antibodies), it seemed much easier for all parties to accept the fact that some agents (e.g., CAR bacillus) were only rarely diagnosed by serology and could be regarded as a true expression of reality.[20]

Although an agent such as *Helicobacter*, which was first described in laboratory rodents in the 1980s, can be cultivated, the introduction of the less costly and less laborious polymerase chain reaction (PCR) made this the method of choice for screening of such new agents,[27] and, therefore, for *Helicobacter*, cultivation techniques never really found a place in routine health monitoring. Although cultivation is still used today as the preferred tool for bacterial screening, PCR or other similar molecular methods will be replacing it for most bacterial infections in future health monitoring of rodent colonies.

1.4 The impact of the symbiotic microbiota

1.4.1 The development of gnotobiotechnology

The rodent gastrointestinal tract (representative of the case for all other mammals) is inhabited by more than 500 species of symbiotic bacteria, depending on how they are identified and categorized. One of Louis Pasteur's hypotheses was that animal life without indigenous microorganisms would be impossible[28]; two other key scientists of that time, Marceli Nencki and Élie Metchnikoff, on the contrary stated it would be longer and healthier.[29,30] To prove either one of these hypotheses from the late nineteenth century until around 1950, eight different groups made attempts to produce germ-free animals.[31] Around the time of World War I, it was clear that animals could live without germs for extended periods, and in the 1930s, Gusta Glimstedt and Bengt Gustaffsson at the University in Lund in Sweden and James A. Reyniers at the University of Notre Dame in Indiana reported the rearing of germ-free animals in stainless steel isolators, thereby developing the discipline of gnotobiotechnology.[32,33] The first screenings of germ-free status were based solely on cultivation and microscopy, but there seemed to be a clear understanding that there might be other microbes present in an abun-

dance too low to allow detection by these means.[34] Today, germ-free rearing is common practice in a range of laboratories all over the world.

1.4.2 Schaedler's flora

Germ-free rodents seem to be predisposed to opportunistic infections when removed from their isolators and introduced into barrier rooms. As just a few bacteria seem to minimize this phenomenon, Russell W. Schaedler from Thomas Jefferson University in Pennsylvania in 1965 isolated and grew bacteria from conventional and SPF mice housed at Rockefeller University in New York.[35] What he found were categorized as lactobacilli, streptococci, *Flavobacterium* spp., coliforms, enterococci, and *Bacteroides* spp.[35] He selected some of the more dominant bacteria that could be isolated in culture, such as two strains of lactobacilli (thought to be *Lactobacillus acidophilus* and *L. salivarius*), one strain of anaerobic streptococci group N (*Streptococcus fecalis*), two strains of *Bacteroides* spp. (one of them thought to be *Bacteroides distasonis*), and one coliform strain (*Escherichia coli).* Subsequently, he fed some of these bacteria to germ-free mice.[36] Germ-free mice associated with this combination could be bred for generations still harboring the flora, and in contrast to germ-free animals, which exhibit a severely enlarged cecum and abnormal intestinal histology, the intestine and cecum of the recolonized mice and their offspring appeared entirely normal.[36] In other combinations, he included a species of *Clostridium,* along with an anaerobic fusiform species,[37] and the cocktail became known as the Schaedler flora.

In 1978, one of his former doctoral students, Roger P. Orcutt, helped the National Cancer Institute in Maryland revise and standardize the Schaedler flora, making it the altered Schaedler flora (ASF).[37] The two lactobacilli, the *Bacteroides*, and the fusiform bacterium remained in the new cocktail, and four new bacteria were added.[37] Precise methods for confirmation and characterization did not come along until later, but today the ASF bacteria are characterized as shown in Table 1.1.[37] The ASF have become the starting flora of many breeding colonies. In the beginning, these were seen as colonization-resistance floras (i.e., it was hoped that they would prevent other microbes from colonizing). However, the majority of the remaining members of the microbiome of a barrier-bred colony are most likely transferred from either humans emitting several microbe-carrying particles per minute[38] or from the diet.[39] Therefore, such a phenomenon as normal flora probably does not exist for these animals because little of it originates from the natural habitat of mice. In two different studies, SPF mice showed a less-complex flora, fewer *Bacteroides* and *Lactobacillus* spp., but more *Clostridia* spp., when compared to either feral or conventional mice, and although similar bacteria were detected, the ASF bacteria were not recovered.[40,41] Other groups, however, have

Table 1.1 Bacterial Species in the Altered Schaedler Flora

Original designation	Systematics
ASF 356	Closely related to *Clostridium propionicum*
ASF 360	Novel *Lactobacillus* spp. clustering with *L. acidophilus* and *L. lactis*
ASF 361	*Lactobacillus* spp.
ASF 457	*Mucispirillum schaedleri*
ASF 492	*Eubacterium pexicaudatum*
ASF 500	A novel unnamed genus in Firmicutes with Bacillaceae and Clostridiaceae relations
ASF 502	*Clostridium* cluster XIV
ASF 519	A novel unnamed genus in Bacteroidetes clustering with *B. distasonis*, *B. merdae*, CDC group DF-3, and *B. forsythus*.

Source: Dewhirst FE, Chien CC, Paster BJ, Ericson RL, Orcutt RP, Schauer DB, et al. *Appl Environ Microbiol* 1999; 65:3287–3292.

observed the colonization of the ASF bacteria even several years after establishing a barrier unit,[42,43] but also that the profile is subject to a substantial cage effect.[43]

1.4.3 Microbiome studies

Cultivation today still seems to be the main practice for screening for germ-free status, maybe because more modern techniques based on molecular biology show too much contamination. However, while the first gnotobiotechnologists were unable to describe more than approximately 15–20% of what could be of potential interest in the microbiome, modern sequencing techniques have allowed full characterization of the microbiome. This has led both to studies in which microbiota-harboring mice have been characterized in relation to the development of specific human diseases, such as type 1 diabetes,[44] type 2 diabetes,[45] obesity,[46] and colitis,[47] and to a new age of germ-free studies in which germ-free mice are recolonized with a microbiota of other mice with a specific phenotype hypothesized to be transferred with the microbiota. One of the pioneers of this has been Jeff Gordon from Washington University, and it was in his laboratory that Peter Turnbaugh in 2006 transferred the obese phenotype of leptin-deficient mice to germ-free wild-type mice in isolators.[48] Today, this has become some sort of revival of Koch's third postulate, that is, to show that a certain phenotype is under impact of the microbiome, gut contents are transplanted to germ-free mice, which afterward are characterized for traits of that phenotype. An alternative approach has been to turn off a specific phenotype by the application of antibiotics.[49–52]

1.5 The future of laboratory animal bacteriology

One of the older bacteria still found in rodent colonies is *Pasteurella pneumotropica*. In 1969, Patricia Brennan showed that it could only induce pneumonia in mice if inoculated together with *Mycoplasma* spp.[53] However, for many years, it was considered a primary inducer of suppurative disease in mice.[54] Along with the evolvement of screenings for viruses and *Mycoplasma* spp., breeders have eliminated these primary pathogens from their colonies, thereby also minimizing previous problems related to *P. pneumotropica*, which obviously has been mostly secondary to other agents. The reason for *Pasteurella*'s "success" as a member of health-monitoring programs is probably because of the combination of its ability to be cultivated and its difficult eradication rather than its impact

Table 1.2 Five Examples of Bacterial Species Newly Recognized for Their Potential to Interfere with Animal Models and Therefore Potential Candidates for Future Membership of Bacteriological Screening Programs

Species	Phylum	Impact on rodent models
Segmented filamentous bacteria (SFBs) or *Candidatus savagella*	Firmicutes	Increased severity of inflammatory bowel disease[1] Decreased incidence of type 1 diabetes[2]
Bifidobacterium spp.	Actinobacteria	Decreased severity of inflammatory bowel disease[3–6] Decreased level of inflammation[7] Decreased level of leptin in rats[8] Decreased allergic sensitization[9] Decreased myocardial infarction[8]
Bacteroides vulgatus	Bacteroidetes	Increased severity of inflammatory bowel disease[10]
Bacteroides fragilis	Bacteroidetes	Increased severity of inflammatory bowel disease[11] Increased incidence of colonic tumors[12] Protection against *Helicobacter hepaticus*-induced colitis[13]
Akkermansia muciniphila	Verrucomicrobia	Decreased severity of obesity and type 2 diabetes[14,15] Decreased incidence of type 1 diabetes[16] Increased susceptibility to allergic asthma[17] Decreased severity of inflammatory bowel disease[18] Higher-severity *Salmonella typhimurium* infection[19] Increased incidence of colonic tumors[20]

Table 1.2 (continued) Five Examples of Bacterial Species Newly Recognized for Their Potential to Interfere with Animal Models and Therefore Potential Candidates for Future Membership of Bacteriological Screening Programs

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on research. Today, as PCR is replacing cultivation as a diagnostic tool, what can be cultivated becomes less relevant and which agents are likely to change the expression of research models more relevant.

Table 1.2 provides some examples of bacterial species for which modern techniques based on PCR or sequencing have revealed a strong potential for influencing animal models.[3,45,55–72] Many of these seem to have a broader and far more serious impact on animal models than many easily cultivable bacteria. Therefore, it is likely as costs of sequencing and multiplex PCRs are rapidly declining that these bacteria will be the future members of routine bacteriological programs in rodent colonies rather than "old enemies" such as *P. pneumotropica*.[73] Their absence will not be the only goal. For some models, they will act in favor of the model; for others, they will act against it. Not only their qualitative association with

the animals (i.e., whether they are to be found or not in the animal) will be of interest but also it will be necessary to consider their abundance. It is also reasonable to assume that a full microbiome characterization in the future will become an important part of the characterizations of many animal models.

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