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From Farming to Engineering: The Microbiota and Allergic Diseases

Dominique Angèle Vuitton [a,b,*], Jean-Charles Dalphin [a,b]

[a] University Bourgogne Franche-Comté, Besancon 25030, France
[b] Allergology Network of Franche-Comté, Besancon 25030, France

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ABSTRACT

The steady increase of IgE-dependent allergic diseases after the Second World War is a unique phenomenon in the history of humankind. Numerous cross-sectional studies, comprehensive longitudinal cohort studies of children living in various types of environment, and mechanistic experimental studies have pointed to the disappearance of "protective factors" related to major changes in lifestyle and environment. A common unifying concept is that of the immunoregulatory role of the gut microbiota. This review focuses on the protection against allergic disorders that is provided by the farming environment and by exposure to microbial diversity. It also questions whether and how microbial bioengineering will be able in the future to restore an interplay that was beneficial to the proper immunological development of children in the past and that was irreversibly disrupted by changes in lifestyle. The protective "farming environment" includes independent and additional influences: contact with animals, stay in barns/stables, and consumption of unprocessed milk and milk products, by mothers during pregnancy and by children in early life. More than the overall quantity of microbes, the biodiversity of the farm microbial environment appears to be crucial for this protection, as does the biodiversity of the gut microbiota that it may provide. Use of conventional probiotics, especially various species or strains of *Lactobacillus* and *Bifidobacterium*, has not fulfilled the expectations of allergists and pediatricians to prevent allergy. Among the specific organisms present in cowsheds that could be used for prevention, *Acinetobacter* (*A.*) *lwoffii* F78, *Lactococcus* (*L.*) *lactis* G121, and *Staphylococcus* (*S.*) *sciuri* W620 seem to be the most promising, based on experimental studies in mouse models of allergic respiratory diseases. However, the development of a new generation of probiotics based on very productive research on the farming environment faces several obstacles that cannot be overcome without a close collaboration between microbiologists, immunologists, and bioengineers, as well as pediatricians, allergists, specialists of clinical trials, and ethical committees.

1. Introduction: Changes in lifestyle and the emergence of allergic/atopic diseases

In the second half of the 20th century, the impressive increase in IgE-dependent allergic diseases—also called "atopic diseases," and including asthma, allergic rhinitis, atopic dermatitis/eczema, and food allergies—became an embarrassing enigma. Since the 1980s, epidemiologists and immunologists have been addressing the questions raised by this unexpected increase, and all studies point to the responsibility of the major changes in lifestyle and environment that occurred in so-called "developed countries" after the Second World War [1–3]. Not all answers have been obtained yet; however, numerous studies (see Refs. [4–8] for a review) have now provided us with a conceptual and operational framework to better understand this unique phenomenon in the history of humankind. Coincidentally, there has been a renewal of interest in the microbiota: the billions of microorganisms that constitute the microflora, and that exert a symbiotic function in the gut of mammals. These microorganisms must definitely be considered as important actors in human homeostasis; their genome, the microbiome, interacts with

* Corresponding author.
E-mail address: dominique.vuitton@univ-fcomte.fr

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the host's genome in many different ways [9,10]. The link that has been established between environmental influences and the gut microbiota in promoting immune tolerance, combined with progress in microbiota engineering, causes us to expect an exciting future for the treatment and/or prevention of allergic diseases. This issue is crucial: Even though the allergy epidemic seems to have now reached a plateau in most countries with a "westernized" lifestyle, it has become a global public health problem in countries with an emerging market economy, as well as in the continually growing cities of low-resource countries [11].

After summarizing the essential historical steps that led to our current understanding of the link between allergies and the microbiota, this review will focus on a particularly puzzling issue that emerged in the 1990s: the protection against allergic disorders that is provided by a farming environment. It will also question whether and how microbial engineering will be able to restore an interplay that was beneficial to the proper immunological development of children in the past and that was irreversibly disrupted by changes in lifestyle. Most of the results come from key cross-sectional studies performed in Europe—especially the Allergy and Endotoxin (ALEX) study [12], the Prevention of Allergy-Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) study [13], and the Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community (GABRIEL) [14]—and from a European five-country case-control birth cohort that was specially designed to elucidate the relationship between farming and allergy—the Protection against Allergy: Study in Rural Environments (PASTURE), in which we have been involved for the past 13 years [7]. Complementary data is found in other studies performed all over the world [15–17] and in comprehensive reviews on allergy prevention using conventional probiotics [18–21]. Although it has become clear in the last few years that the microbiomes of the upper respiratory tract, skin, and lung, as well as that in breast milk, which was long considered to be sterile, are involved in the genesis and/or manifestation of allergic conditions [22–27], the cause-effect relationships are less well elucidated; thus, these relationships will not be taken into account in this review, which will focus on the gut microbiome.

2. Risk factors for the development of allergic diseases: Genes versus environment

2.1. Genetic factors

The family/hereditary nature of a series of disorders, including asthma, hay fever, and other types of allergic rhinitis, atopic dermatitis, urticaria, and food allergy, was first introduced in 1923 by Coca and Cooke [28], who proposed the common denomination of "atopic diseases." It is now accepted that a multifactorial determination combines genetic and environmental factors. Common genetic determinants operate for all diseases associated with an excessive or inappropriate IgE antibody response toward environmental antigens, and specific genetic determinants operate for the various clinical conditions [29,30]. Of allergic children, 50% have an allergic family history if grandparents are considered [31]; a tendency toward the same type of clinical manifestation in monozygotic twins also provides evidence of the genetic nature of atopy [32]. Recent genome-wide association studies have uncovered several novel genes underlying asthma, including single-nucleotide polymorphisms in *IL18R1*, *IL33*, *SMAD3*, *ORMDL3* (corresponding to variants on chromosome 17q21 and specific to childhood-onset disease), *HLA-DQ,* and *IL2RB* loci [33]. Most asthma/atopy genes are not replicable across populations because of differences in the epidemiology of these genes, as may be observed between Chinese subjects and subjects from other ethnic groups [34]. Studies on the gene polymorphisms of *ORMDL3* at chromosome17q21 somehow gave discordant results in different Chinese populations, although recent studies have shown that these polymorphisms were actually associated with childhood-onset asthma in the Han population of Northeast China, as found in Caucasian children [35].

The genes controlling IgE levels have been found to have little overlap with the genes mediating asthma susceptibility; the former are more directly involved in the "atopic" background [36,37]. The atopic—or IgE-dependent—immune profile is immunologically characterized by a predominance of type 2 T-helper cell (Th2) immune response, including the secretion of interleukin (IL)-4, IL-5, and IL-13, a profile that is also observed in helminth infections and in fetal life. This is in contrast to the type 1 T-helper cell (Th1) profile, which is dominated by the secretion of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interferon (IFN)-γ, and which is adapted to fighting against bacteria and intracellular infectious agents [38]. Nevertheless, it may be kept in mind that 23% of children without any family disposition also develop asthma and/or allergy [19], and that this percentage is likely to increase with time in those countries where the incidence of allergy is still on the ascending slope.

2.2. Personal history and environmental factors

It is now well established that environmental factors play a major role both in the development of allergic sensitization and in the clinical expression of disease. As an example of family/personal environmental risk factor, maternal tobacco smoking is well recognized [39], and recently identified genes underlying asthma have been shown to interact with *in-utero* and early-life tobacco smoke exposure [40]. The increase in allergy incidence in western/northern Europe and in the US and the difference observed with "developing countries" were initially attributed to better diagnosis, as well as to new contacts with allergenic substances that were not or little encountered in the past. It soon appeared, however, that the incidence of allergic diseases might also be markedly different between "developed" regions/countries with a similar level of healthcare management, between urban versus rural environments, and/or between wealthier versus less wealthy regions/countries. A similar observation had already been made by a 18th century English family doctor who had stressed that, despite their usual contact with hay, farmers' children suffered extremely rarely from the seasonal hay fever observed in their rich and noble counterparts [41]! Comparisons between allergy prevalence in regions with different levels of development in China (e.g., Hong Kong, Guangzhou, and Beijing; or Hong Kong, Beijing, and Urumqi) and between the incidence of allergic diseases in the first versus the second generation of immigrant populations from developing to developed countries have fully supported the role of lifestyle changes, irrespective of the genetics of the populations [15–17,42–45]. Epidemiology research in the 1980s and 1990s globally ruled out the responsibility of air pollution in the increased incidence of allergy and confirmed its responsibility in the severity of respiratory clinical symptoms [46]. Studies on breast feeding and/or food diversification in the first year of life provided rather non-conclusive results, which are summarized in reviews and meta-analyses [47,48].

Cross-sectional studies performed in the 1980s and 1990s stressed a series of environmental situations that could explain the "post-industrial revolution epidemic" of allergies. For example, the "protective effect" of a high number of siblings was the origin of the popular "hygiene hypothesis" proposed by Strachan in 1989 [49], which was further supported by similar observations in other countries [50,51] and by the protective effect of early-life day-care attendance and of common viral infections of childhood such as hepatitis A, measles, or *Toxoplasma gondii* infection [52–55].

Since that time, the paradigm has been constantly revisited, and has moved from mere "hygiene" and "lack of immune stimulation by infectious agents" to immune modulation by nonpathogenic microbial experience [56,57] (Fig. 1), such as the association of the risk to become allergic with a cesarean section as the mode of delivery and with antibiotic treatments of the pregnant mother and/or infant [58,59]. Finally, the paradoxical protective effect of parasitic (especially helminth) infections—which, however, share a similar Th2 immunological profile with allergic diseases—completed the picture by focusing attention on situations that may install or restore immune tolerance, rather than on those that only establish an anti-microbial Th1 response [60,61] (Fig. 1).

3. Farming environment and protection against allergy

3.1. Epidemiological observations

Most of the European studies on the farming environment started from observations made after the dissolution of the Soviet Union and in the first years of German reunification (i.e., at the beginning of the 1990s). A lower prevalence of allergic diseases was observed in children living in the eastern part of Germany compared with the western part (18.2% versus 36.7% for atopic sensitization; 3.9% versus 5.9% for asthma and hay fever) [62], a difference that was "corrected" a few years later, after the reunification of Germany [63]. A lower incidence of atopy was also reported in children living in Russian compared with Finnish Karelia, as well as in Estonia and Poland compared with Sweden [7,64–67]. Complementary studies on the influence of the mode of heating in rural communities in southern Bavaria [68] caused a research team based in Munich, Germany, to suggest that the use of home wood- or coal-burning stoves, which were found to be associated with fewer allergic diseases, might be a surrogate for a more traditional lifestyle and for farming as the occupation of the parents [68]. A comparison between farming and non-farming families fully confirmed that suggestion [69]. From these results, and from the results of studies performed in Austria, Switzerland, Finland, and Canada at the end of the 1990s (reviewed in Refs. [4,70–75]), it became clear that protective factors were operating in less-developed rural environments [1]. Results of the ALEX study confirmed that protection was more pronounced when traditional farming and style of life were preserved [12]. More recent studies [76,77], as well as the dedicated PARSIFAL and GABRIEL cross-sectional studies and the PASTURE cohort [7,13,66,67], have totally confirmed the unique role of a farming environment. A list of studies on farming and the risk of developing allergies that were performed until 2010 is available in the excellent review by von Mutius and Vercelli [70]. Possible differences in lifestyle between farmers and non-farmers, such as maternal tobacco smoking, duration of breast feeding and other dietary habits, day care, pet ownership, family size, parental education level, and family history of allergy, did not account for the protective "farm effect"; all confounding factors were carefully ruled out by the statistical analyses [70]. Independent protection factors against occurrence of allergies in children that were consistently identified by the various studies included: ① stay in barns and stables, and animal exposure; and ② consumption of raw, unpasteurized milk. Exposure of both mothers during pregnancy and children in their early life was responsible for the protection (Fig. 1).

3.2. Role of barn/stable environment and animal exposure

The most comprehensive studies dedicated to disentangling the various factors of the protection against allergy provided by farming, such as ALEX, GABRIEL Advanced Surveys, and PASTURE, have been performed in European regions where dairy production is the main activity and where farming is not industrialized; that is, in mid-mountain-altitude cheese-production areas of the Alps and Jura [78,79]. All aspects of the farming activities, as well as the characteristics of the farms and their environment, were carefully recorded and specific environmental samples (endotoxins, allergens, Gram-positive and Gram-negative bacteria, molds, and fungi) were collected in stables, barns, and family homes, including house dust and mattress dust in the mother's and infant/child's bed. Specific nested studies were also performed to better identify the microbial environment of the farms according to their architectural and geographical characteristics and farming activities, and to identify

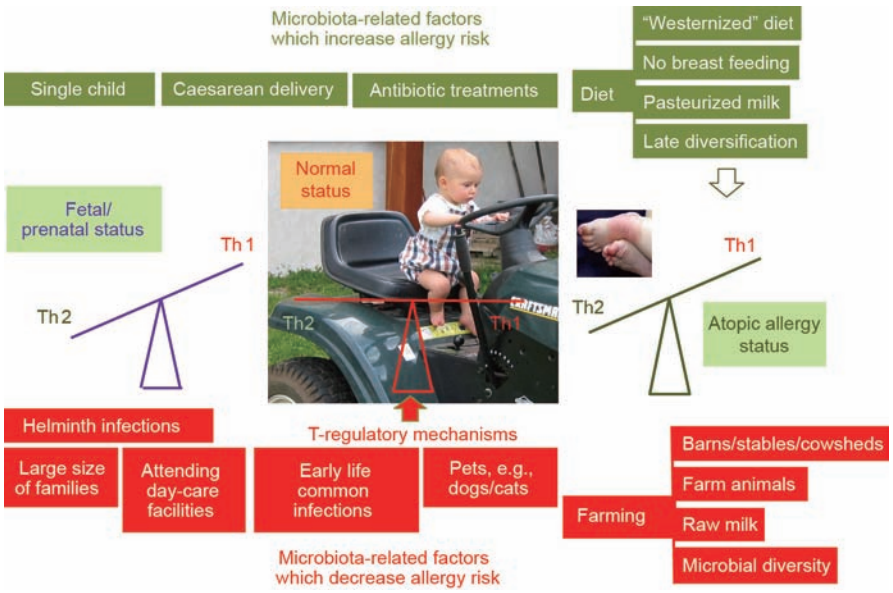

Fig. 1. Microbiota-related factors that may either increase or decrease the risk of allergic disorders in a child. Elements of the immune balance are voluntarily simplified. T-regulatory mechanisms include: the shaping of adaptive immunity by innate immunity, and especially by the intervention of Toll-like receptors (TLRs), dendritic cells, and related cytokines (IL-12, TNF-α, etc.); the suppression of Th2 cells and cytokines (IL-4, IL-5, IL-13); and the induction of the various populations of T-regulatory cells and of regulatory cytokines (IL-10, tumor growth factor (TGF)-β, etc.).

the microbial and chemical composition of the collected farm milk [7,14,80].

Exposures associated with a farming lifestyle include the length of stay in barns and/or stables and contact with farm animals, mostly cattle, pigs, and poultry (with an occasional contribution of exposure to cats and dogs) [54]. From the ALEX study, the exposure of children aged younger than 1 year to stables, compared with those aged 1–5 years, was associated with lower frequencies of asthma (1% vs. 11%), hay fever (3% vs. 13%), and atopic sensitization (12% vs. 29%); protection against asthma was independent from protection against atopic sensitization [12]. In the same study, the inverse relation between current dog contact, asthma, and allergy was explained by simultaneous exposure to stable animals, or was restricted to farm children [81]. Exposure to barns and stables and/or farm animals of the mothers during pregnancy was also shown to be crucial [82]. In another study in Germany, the risk reduction was stronger for children whose families were running a farm on a full-time basis as compared with families with part-time farming activity [69]. In a stratified random subsample of 8419 children in phase II of the GABRIEL Advanced Surveys, children living on a farm were at a significantly reduced risk of asthma, hay fever, atopic dermatitis, and atopic sensitization compared with non-farm children; the overall "farm effect" was explained by specific exposure to cows and contact with straw for asthma, and exposure to fodder storage rooms and manure for atopic dermatitis [83].

Pregnancy and the first year of age were often considered to represent a window of opportunity; however, continual long-term exposure to stables until age 5 was associated with the lowest frequencies of asthma, hay fever, and atopic sensitization [11]. Other studies have shown that long-term exposure can also be beneficial: In a cross-sectional study of more than 2500 families, the combination of current and childhood exposure was more strongly negatively associated with shortness of breath, wheezing, use of asthma medication, and asthma than childhood exposure alone or current exposure alone. Moreover, the combined number of years of farm exposure in childhood and adulthood showed a dose-dependent inverse association with symptom prevalence [84]. A recent study in the US has shown that the influence of maternal farming and exposure to farming in early childhood can still be observed in adults [85].

The results of PASTURE and associated studies have provided novel and interesting immunological correlations to exposure to barns, stables, and animals, especially regarding the specific influence of the mother's environment during pregnancy, an influence that adds to the genetic predisposition [33] and interferes with the atopic status of the mother, an immunological profile associated with an impairment in regulatory T cells (Tregs) [86]. An inverse relationship was observed between the level of IgE antibodies and seasonal allergens in newborns' cord blood and maternal exposure to animal sheds during pregnancy [66], while a direct relationship was found between Th1 cytokines in cord blood (which represent an immunological profile that is non-prone to allergy development) and contact of the mother with a variety of animals [67]. Farm exposure during pregnancy increased the number and function of cord blood Tregs associated with lower Th2 cytokine secretion and lymphocyte proliferation; the positive correlation between Th17 lineage markers and FOXP3 mRNA (a marker of a subset of Tregs) found in cord blood after non-specific cell stimulation was also influenced by maternal farming [87]. Treg percentage before and after stimulation and FOXP3 mRNA expression *ex vivo* as well as mRNA expression of Th1/Th2/Th17-associated cell markers then decreased from age 4.5 to 6; animal/stable exposure was also associated with decreased lipopolysaccharide (LPS)-stimulated Treg percentage at age 6 [88].

The level of environmental exposure to endotoxins (LPS) was first proposed to be "the" protective determinant against the development of atopic diseases in childhood [89]. In ALEX, endotoxin concentrations were found to be the highest in stables, and higher indoors in the dust from kitchen floors and children's mattresses of farming families than in those of non-farming families. Endotoxin levels were also significantly higher in mattresses and in the dust from kitchen floors in households where children had regular contact with farm animals [5,80,90]. However, based on different studies, it now appears that diversity of exposure, including several types of bacteria, actinomycetes, molds, and other types of fungi, is more important than a single bacterial component or even than the total microbial load in farms [70,89].

Contacts with hay, straw, fodder, and silage have also been stressed as protective factors. Helping with haying was the only variable related to a farming environment that had a consistent inverse association with both current symptoms and a doctor's diagnosis of atopic dermatitis in the PARSIFAL study [91]. A nested study within the PASTURE project showed that indoor pollen in barns and stables was significantly higher in winter than in summer and exceeded by far the typical outdoor levels during the pollen season [78], thus suggesting that the inhalation of high quantities of pollen by children during the period of maturation of the immune system could contribute to establishing immune tolerance. Fungal agents and actinomycetes cannot be ruled out as part of the exposure to hay/fodder, as well as bacteria for silage, all of which could participate in the "farm effect" [92]. Organisms are transported from animal sheds and barns into farm dwellings, thus adding to the direct exposure to barns and stables [79].

The protective influence from exposure to a farm environment is not restricted to dairy farming and European farms; a few studies have also shown a positive association between living in a crop-farming family during the first year of age and protection against asthma. One such study was performed in Guangdong Province, China, where asthma protection was also associated with endotoxin level in house dust [93]. However, available information on the possible microbial and non-microbial candidates to support the "farm effect" in this situation is very scarce.

3.3. Role of raw milk consumption

In continental Europe, as well as in the UK, Crete of Greece, and New Zealand, epidemiological studies have provided evidence for an unexpected but indisputable link between allergy protection and drinking raw, unpasteurized milk during pregnancy for the mother and during the first year of life for the infant [72,94,95]. In ALEX, drinking unpasteurized milk during pregnancy and the first year of life resulted in a significantly lower prevalence of asthma (0.8% vs. 11.8%), hay fever (0.8% vs. 16.0%), and atopic sensitization (8.2% vs. 32.9%). This effect was independent from and synergistic with exposure to barns and/or stables and farm animals, and remained significant after adjusting for potential confounders. PARSIFAL and GABRIEL, which included a more detailed analysis of the environment and diet, have provided evidence for a specific and independent influence of raw milk consumption in the protection against allergy [96,97]. The effect of drinking raw milk was independent from the family allergy history, and the influence of raw milk consumption was also true for non-farm children [96], an observation that had already been made in previous studies [98,99]. In PASTURE, the above-mentioned elevation of Th1 cytokines in cord blood was also and independently associated with raw milk consumption by the pregnant mother, with an additive effect of raw milk-butter consumption [67]. An association with raw milk consumption by the mother during pregnancy was also found for Tregs in cord blood; FOXP3 demethylation was increased in the offspring of mothers who drank farm milk, suggesting epigenetic changes with consequences for immune tolerance through Treg modulation [100]. In addition, in children aged 4.5 years, raw milk exposure was independently associated with

increased Treg cell numbers on stimulation and with higher FOXP3 demethylation [87]. Clinical relevance was shown by the reduction of the risk of asthma at 6 years of age by the previous consumption of unprocessed farm milk, compared with shop milk [101].

As mentioned above, endotoxins from Gram-negative bacteria were the first candidates for protection; however, raw milk collected from the PASTURE/Mechanism of Early Protective Exposures on Allergy Development (EFRAIM) study farming families did not contain more endotoxins than the milk collected from non-farming families [102]. In later studies, β-(1→3)-glucans, extracellular polysaccharides, and muramic acid from molds and Gram-positive bacteria were associated with a reduced risk of allergy and a reduced risk of asthma in rural and urban populations [94]. Experimental studies also confirmed that the anti-allergic biological activity of stable dust extract was not exclusively mediated by endotoxins [103]. In more general terms, the diversity of microbial exposure, both fungal and bacterial, is associated with a reduced risk of allergy in farmers' children [104]. Biodiversity is actually a particularity of the microbiota present in raw milk and raw-milk products [105]. Candidate fungi, including *Absidia* spp., *Eurotium* spp., *Cladosporium* spp., *Penicillium* spp., as well as mesophilic actinomycetes, may be transported from animal sheds and barns into dwellings, but may also be present in milk [79,92]. Although a variety of organisms were found in the raw milk studied in the GABRIEL Advanced Surveys, including micrococci and staphylococci, lactobacilli, bacilli, other bacterial endospores, and psychrotrophic bacteria, the total viable bacterial counts or bacterial subgroups in farm milk were not significantly related to asthma or atopy [97]. This finding suggests that non-viable bacteria are likely to be involved in the immunomodulating effect, and/or that non-bacterial organisms also play a role; it also suggests that non-microbial components may be involved. These non-microbial components, which are expressed differently in pasteurized milk than in raw milk, could thus interfere, in a natural "prebiotic" way, with the immune response and, consequently, with the development of allergy; such components are currently the subject of active research.

The reduced risk of asthma that was observed in PASTURE in children at 6 years of age was partially explained by the fat content of raw milk, which has higher levels of ω-3 polyunsaturated fatty acids [101]. However, when mice were fed a diet that was rich in milk conjugated linoleic acid (CLA), this diet neither reduced the hallmarks of allergic airway inflammation in sensitized and ovalbumin-challenged mice nor modified the eicosanoid pattern in the bronchoalveolar lavage fluid of these animals, even though the CLA levels were elevated in the plasma and erythrocyte membranes [106]. On the other hand, increased levels of the whey proteins bovine serum albumin, α-lactalbumin, and β-lactoglobulin were inversely associated with asthma but not with atopy [97]. Other components may also be involved, such as vitamin D, since it was shown in PASTURE that maternal vitamin D supplementation during pregnancy was associated with an increase in the gene expression of immunoglobulin-like transcript (ILT)3 and ILT4, two markers of tolerogenic presentation of antigens [107]. Whether these findings may be extrapolated to raw-milk cheeses is as yet unverified; however, the very high diversity of the microflora in raw-milk cheeses, as well as the changes that occur in other components during ripening, may cause such cheeses to also play a role in the maintenance of a well-balanced immunity [105]. Whatever the components of raw milk may be that are involved in the protective effect of raw-milk products, their mode of action has yet to be elucidated.

4. The microbiota and allergy

4.1. The intestinal microbiota and the induction of tolerance

Back in the 1970s, the development of the model of germ-free and gnotobiotic mice—that is, mice reconstituted with a specific gut microflora [108,109]—along with studies on the lymphoid reconstitution of fetal grafts of intestinal mucosa and Peyer's patches, allowed immunologists to understand that the gut microflora was a key player in the development of gut-associated lymphoid tissue (GALT) and in the balanced immune response to both microorganisms and foreign proteins [110–113]. After more than 30 years of near-oblivion, and due to the genomic revolution in microflora/microbiota/microbiome research since the beginning of the 21st century, the central role of the microbiome as a "metagenome" of mammals has been revealed; not only in all aspects of the immune response, its first recognized playground, but also in a variety of metabolisms and interactions aimed at establishing and maintaining homeostasis. The combination of high-throughput methods with 16S ribosomal RNA gene analysis has allowed researchers to avoid the constraints and biases of microbial culture to fully characterize and classify the 100 trillion microbes from the four main phyla, Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria, that have shaped our immune system due to a symbiotic relationship and evolution. Numerous reviews encompass the multiform and always growing interferences of the microbiota in health and diseases [10,58,59,114].

The intervention of the microflora in the development of the immune system has long been known, and its intervention in immune tolerance has long been suspected [111,113]. Microbial signals are responsible for the induction and development of isolated lymphoid follicles in the intestinal tract, and also for the constitution of the intestinal barrier through epithelial cell maturation and angiogenesis; these processes involve a variety of pattern-recognition receptors (PRRs) that are capable of detecting microbe-associated molecular patterns (MAMPs), including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) [115]. Controlling the primary encounter with pathogens also involves the mucus layer and antimicrobial peptides, which are both under the tight control of the microbiota; the engagement of PRRs by microbiota-derived products induces the expression of a variety of antimicrobial peptides that are critical in preventing the translocation of bacteria through the rest of the host tissue. These peptides include RegIIIγ lectin, which is expressed soon after birth or following the colonization of germ-free mice and which has a microbicidal effect on Gram-positive bacteria, or α-defensins and cryptdins, which are produced by Paneth cells under the control of PRR NOD2 [116,117].

Another means by which the microbiota may control the host's tolerance toward ingested microbes/antigens is associated with the role of the microbiota in the control of the antigen sampling of luminal contents by dendritic cells from the underlying lamina propria compartment. Recent reports indicate that the gut microbiota can directly contribute to the expansion of lamina propria resident CX3CR1 macrophages that have been associated with the local expansion of Tregs; nevertheless, the influence of the microbiota on microfold cells that are specialized in antigen transfer and on mucosal antigen-presenting cell function clearly deserves further study [118]. The microbiota is also crucial for the development and epitope recognition of secretory IgA (sIgA), which is the main type of immunoglobulin produced by B cells of the GALT, and which is secreted in the intestinal lumen across the mucosal epithelium. sIgAs are involved in trapping bacteria and unwanted proteins in the mucus layer, thus preventing allergen systemic uptake and allergenic sensitization. IgAs, before their binding to the polymeric Ig receptor at the basal membrane of gut epithelial cells, may also be involved in preventing food allergies by preventing allergen access to specific IgEs bound on mucosal mast cells [119].

The gut mucosa is the first line of interaction between mammals and both microbial and non-microbial life-threatening components of the environment [120]. In order to prevent harmful reactions to

otherwise harmless foreign components, such as dietary and other environmental proteins, the microbiota controls the subtle balance between inflammatory and regulatory response that is orchestrated by T cells, by tightly regulating the production of cytokines. Germ-free mice show extensive deficiencies in basal cytokine production, and in the absence of the microbiota, the cluster of differentiation (CD)4^+ T-cell population is reduced, disproportionately affecting Th1 and Th17 cells. Colonization of germ-free mice with a complex microbiota restores a broad spectrum of Th1, Th17, and Treg responses [121,122]. The existence of oral tolerance has been known for decades; however, its mechanisms and dependence upon the gut microbiota were only recently partially elucidated, by taking advantage of a better knowledge of Tregs and cytokines [123]. In fact, tolerance induction is the default immune pathway in the gut. The type of tolerance that is induced relates to the dose of antigen that is fed—anergy/deletion for high dose, or Treg induction for low dose. Feeding germ-free mice with bacterial LPS is sufficient to restore the process of oral tolerance [109]; in addition, the presence of the microbiota has been associated with the suppression of IgE and Th2 responses following antigen feeding [22]. However, the precise molecular mechanism that accounts for the role of endotoxins and their cross talk with TLR4 in the phenomenon of oral tolerance and the targets of microbe-derived signals remain incompletely understood [110]. Some unexplained discrepancies in experiments with endotoxins parallel the ambiguous role of endotoxins in the development of allergy in early childhood [89]. The conditioning of gut dendritic cells by gut epithelial cells and the gut microbiota induces $CD103^+$ retinoic acid-dependent dendritic cells, which in turn induce Tregs and their various cytokine secretions at mucosal surfaces. Th3-type Tregs were discovered in the context of oral tolerance; they are tumor growth factor (TGF)-β-dependent, and express latency-associated peptide (LAP) on their surface. Type 1 Tregs (Tr1s) are induced by nasal antigens and are IL-10 dependent; in addition, $FOXP3^+$-inducible Tregs are induced by oral antigens and by the oral administration of aryl hydrocarbon-receptor ligands [123]. Retinoic acid now appears to be a major regulator of the Treg system. However, the precise factors that govern the activation of the enzymes involved in the metabolism of retinoic acid, as well as the processes by which the microbiota or pathogen organisms modify the metabolism of vitamin A, remain poorly understood. Interaction with microbial products via TLR2 can promote vitamin A metabolism; a reciprocal regulation between the microbiota and vitamin A metabolism is further supported by the observation that vitamin A deficiency leads to a dramatic shift in the microbiota [124,125].

4.2. The intestinal microbiota and the emergence/prevention of allergy

The major immunological role that is now attributed to the gut microbiota, the types of immune responses that are associated with allergic/atopic diseases, and the involvement of innate immunity (especially TLRs), Tregs, and cytokine-related regulatory mechanisms in the prevention of allergic sensitization and reactions highly suggest that pre- and immediately post-natal environmental influences may be mediated by modifications of the gut microbiota. Microbiotas that are present in other sites that are targets for the clinical manifestations of allergic diseases, such as the upper respiratory tract, lung, or skin microbiotas, could also play a role [24,126]. As described above, the epidemiological studies were very careful to rule out biases and confounding factors; however, the additive or synergistic effects of several protective factors present in the farm environment and/or "dairy-farming lifestyle" are stressed in several studies. These include the combination of farm-specific exposures to farm animals and raw milk and/or raw-milk products with the following factors: mode of delivery; number of siblings; history of infection in mother and child; antibiotic treatments; exposure to pets and indoor allergens, including dust mites in dwellings and pollen in barns; and dietary components such as breast feeding, early food diversification, and regular consumption of fermented foods (Fig. 1) [54,81,127,128].

It is obvious that these factors, individually or taken together, may interfere with the composition of the gut microbiota [129,130]. Vaginal delivery represents the first encounter with microbial populations that will colonize the newborn's gut to ensure its immunoregulatory functions; caesarean section delays the development of the child's gut microbiota, while shaping it to maternal skin-microbiota patterns [131]. As antibiotics may considerably reduce the total microbiota population at any site, and may durably modify the ratio of the microbiota's components [132,133], antibiotic treatments of the mother during pregnancy and of the newborn, and particularly of hospitalized premature infants, are prone to modify the gut microbiota in a way that prevents the establishment of immune tolerance [134]. The composition of the microbiota then changes substantially from infancy to adulthood, and becomes dependent on inhaled and ingested microbial components of the environment as well as on non-microbial dietary components, which may nevertheless influence gene categories [58,59,114,118]. In addition to the influence of raw milk consumption, it was observed in PASTURE that early diversity of complementary food in the first year of life was associated with a reduction in the risk of having atopic dermatitis with onset after the first year of life, and that the introduction of yogurt in the first year of life also reduced the risk for atopic dermatitis. Unfortunately, the gut microbiota composition in these children was not reported [135].

Before the advent of high-throughput DNA sequencing technologies, studies using the culture of feces and/or detection of specific microbial metabolites showed differences in the intestinal microflora of children living in countries with marked differences in allergy prevalence [136]. Differences were also reported in children who became allergic compared with those who did not [137–140], and in children in a combination of both situations [141]. Such differences were attributed to the children's different diets, and especially to the consumption of more traditional food (including raw and fermented milk, cheeses, and other fermented foods), exposure to endotoxins, life in larger families, less use of antibiotics in non-allergic children, and/or less use of antibiotics in children from low-risk countries and/or their mothers [138,142–144]. The importance of the diversity of the microflora composition and of its changes with time was already stressed in these pioneering studies, and was confirmed in more recent studies using barcoded 16S rDNA 454-pyrosequencing in stool samples at 1 week, 1 month, and 12 months of age in 20 infants with IgE-associated eczema and in 20 infants with no allergic manifestation until 2 years of age [145]. Similar observations were made for asthma; however, there were no significant differences in the relative abundance of bacterial phyla and genera between children with or without allergy [146]. In another study by the same team, reduced diversity of the gut microbiota during infancy was associated with increased risk of allergic sensitization, allergic rhinitis, and peripheral blood eosinophilia, but not with asthma or atopic dermatitis, in the first 6 years of life [147]. In a collaborative international study, children attending Steiner schools (which operate under an anthroposophic lifestyle) had a significantly higher diversity of microbes in their feces than farm children, who in turn had higher diversity than the control groups [148]. Large differences were found in the lactic acid bacteria (LAB) subpopulations in the sampled groups; in some children, the LAB subpopulation was dominated by a species that has not yet been cultivated. The diversity of exposures, more than the total amount of microbes inhaled or ingested, is essential to shape the microbiota properly, and in this respect, the farming environment represents a model of biodiversity [22,104,149].

4.3. Understanding immunological mechanisms in the cross talk between the microbiota and immunological determinants of allergy

Notwithstanding all theoretical arguments that link the farming environment and the microbiota, and the convincing results from well-designed epidemiological and immunological studies, it is clear that the different technologies and study designs used in the various studies currently prevent us from delineating the "good" microbiota that would ensure an infant or child's protection against the further development of allergic diseases. In addition, links between allergic predisposition and the microbiota rely more often on associations than on direct demonstration of a cause-effect relationship, and little is known regarding the molecular mechanisms that may direct the sequence of events from exposure, changes in the composition of the microbiota, and their immunological consequences.

The first set of arguments comes from the regularly observed involvement of qualitative or quantitative changes in TLR and the associated receptors of innate immunity in the protection of children by farming, and from the close link that has been recognized between the microbiota and innate immunity. The ALEX study first pointed to the relationship between farming and receptors of innate immunity—especially TLR2, TLR4, and CD14—both quantitatively (expression level) and qualitatively (polymorphism) [150,151]. More recent results on caspase activation and recruitment domain 4 (CARD4)/NOD1 showed that a strong protective effect of a farming environment on allergies was only found in children who are homozygous for the T allele in *CARD4/-21596*, and not in children who carry the minor allele (C). Among the former, farmers' children had a significantly lower frequency of sensitization against pollen, hay fever, and atopic asthma symptoms than children not living on a farm [152]. Conversely, no significant difference in the prevalence of these phenotypes by farming status was found among children with a C allele in *CARD4/-21596*. Both atopic sensitization and the gene expression of *TLR2*, *TLR4*, and *CD14* were strongly determined by maternal exposure to stables during pregnancy, whereas current exposures had much weaker or no effects. A dose-response relation was found between the extent of up-regulation of these genes and the number of different farm animal species the mothers recruited in PARSIFAL had encountered in their pregnancy [153]. The protective effect of farm milk consumption on allergic diseases was also stronger in children carrying the A allele in *CD14/-1721* than in children who were homozygous for the G allele [154]. In PASTURE, the gene expression of innate immunity receptors in cord blood was higher in the neonates of farmers—significantly so for *TLR7* and *TLR8*—and unboiled farm milk consumption during the first year of life showed the strongest association with TLR4, TLR5, and TLR6 mRNA expression in the first year. The previously described modification of the association between farm milk consumption and *CD14* gene expression by the single nucleotide polymorphism *CD14/C-1721T* was, however, not found [155]. The risk of atopic dermatitis was reduced by more than half among children with mothers having contact with three or more farm animal species during pregnancy, and elevated expression of TLR5 and TLR9 in cord blood was associated with decreased doctor diagnosis of atopic dermatitis. In addition, a significant interaction between polymorphism in *TLR2* and prenatal cat exposure was observed in atopic dermatitis [135,156]. Finally, sIgA levels in the breast milk of the mothers enrolled in the PASTURE cohort were associated with environmental factors related to microbial load; for example, they were associated with contact with farm animals or cats during pregnancy, but not with raw-milk consumption. Furthermore, sIgA levels were inversely associated with atopic dermatitis up to age 2 [157]. Taken together, these results strongly suggest a subtle interplay between farming exposure (including exposure to animals and raw-milk consumption) and individual characteristics of the innate and sIgA immune response very early in life, when the intestinal microbiota is known to play a major role [158,159].

The second set of arguments comes from the early installation of immune regulatory mechanisms, including dendritic cells, Tregs, and regulatory cytokines, and of the Th1 response in children exposed to a farming environment [8]. Most of these immunological observations have already been reported above [56,86–88,100]. Additional confirmation has come from the study of the Finnish cohort within PASTURE, which showed that the unstimulated mononuclear cells of farm children produced more IL-10, IL-12, and IFN-γ than those of non-farm children, and that specific farm exposures were associated with a higher spontaneous production of cytokines [160]. The number of specific farm exposures tended to be dose-dependently associated with a higher spontaneous production of IFN-γ and a lower LPS-induced production of TNF. Observations made from this same subgroup of children indicated a link between innate and adaptive immunity, by characterizing the dendritic cells of farm versus non-farm children: At age 4.5, asthma was positively associated with CD86 expression on myeloid dendritic cells (mDCs) *ex vivo* and inversely associated with IL-6 production in mDCs after stimulation with LPS. LPS stimulation resulted in a lower percentage of mDCs in the cell cultures from farm children, which suggests that farm exposure may have immunomodulatory effects by decreasing the percentage of mDCs [161]. In the same children at age 6, the percentage of $BDCA3^+$ high type 2 mDCs (mDC2s) was lower in farm children; similar associations were found between mDC2 percentage and prenatal and lifetime exposure to farm milk and to stables, although these associations were not independent from farming [162]. A complementary—and independent—effect of the diet was also suggested from the results obtained in PASTURE: Increased diversity of complementary food introduced in the first year of life was inversely associated with asthma with a dose-response effect, and a similar effect was observed for food allergies and food sensitization. Furthermore, increased food diversity was significantly associated with an increased expression of FOXP3 and a decreased expression of Cε germline transcript, which codes for the heavy chain of IgE [163]. The capacity of commensal bacteria such as *Clostridium perfringens* (*C. perfringens*), *Staphylococcus aureus* (*S. aureus*), *Lactobacillus rhamnosus, Escherichia coli* (*E. coli*), and *Bacteroides fragilis* to interfere with neonatal cord blood monocytes or dendritic cells was tested *in vitro*. The Gram-positive bacteria *C. perfringens* and *S. aureus* induced the release of soluble CD14 (sCD14) from monocytes, while Gram-negative bacteria did not. However, both Gram-positive and Gram-negative bacteria induced the release of sCD14 by dendritic cells. In turn, sCD14 and sCD83 inhibited birch pollen allergen-induced Th2 differentiation by suppressing IL-13 production [159]. Another *in vitro* experiment using *Bifidobacterium adolescentis, Enterococcus faecalis, Lactobacillus plantarum* (*L. plantarum*), *Streptococcus mitis, Corynebacterium minutissimum, Clostridium perfringens, Bacteroides vulgatus, E. coli, Pseudomonas aeruginosa, Veillonella parvula,* and *Neisseria sicca* strongly suggested that different bacterial strains have differential effects on the maturation of the immune system of infants [158]: Gram-positive bacteria induced higher levels of IL-12 and TNF-α than Gram-negative bacteria in both cord and adult cells, but Gram-negative and Gram-positive bacteria induced similar levels of IL-6 and IL-10 in cord cells. *L. plantarum* signaled through CD14, TLR2, and TLR4, whereas *E. coli* acted mainly through CD14 and TLR4 [158]. Early induction and sustained maintenance of regulatory mechanisms that are known to be greatly influenced by the gut microbiota strongly suggest the intervention of the latter in the promoting effect of the farm environment.

5. Microbiota engineering to prevent/treat allergic diseases

5.1. Searching for "farming substitutes": The possible use of probiotics

Thus far, probiotics, that is, live microorganisms which when

ingested in adequate amounts confer a beneficial effect on the host, represent the main line of preventive strategies used to modulate the gut microbiota during pregnancy and the first months of life in order to prevent the occurrence of allergic disorders. In 2001, Kalliomäki et al. [164] launched their first prospective double-blind randomized trial using *Lactobacillus* GG in pregnant mothers and infants at high risk of atopy after they found different microbiotas in allergic versus non-allergic children in Finland [140]. The objective of this review is not to analyze the more than 40 clinical trials of probiotics, as recent and excellent reviews and meta-analyses are available. The review by Forsberg et al. [20], the meta-analysis by Zuccotti et al. [165], the meta-analysis by Cuello-Garcia et al. [166], and the meta-analysis by Zhang et al. [167] provide details on selected trials. Probiotics, essentially *Lactobacillus* of various species, or *Lactobacillus* and *Bifidobacterium*, exert no significant prevention of allergic respiratory diseases; however, they lead to a significantly lower relative risk for atopic dermatitis when compared with a placebo. This effect is most pronounced when a combination of probiotics is used and/or when a combined perinatal intervention is undertaken [20]. Such conclusions were used by the World Allergy Organization (WAO)-McMaster University Guidelines for Allergic Disease Prevention to determine that "there is a likely net benefit from using probiotics resulting primarily from prevention of eczema" [19]; however, the Guidelines also confessed that "all recommendations [were] conditional and supported by very low quality evidence." The low and very low certainty of the evidence prompted the Cochrane groups [18] not to recommend the use of probiotics in any circumstances [168].

Attempts at using prebiotics, that is, selective ingredients that allow specific changes both in the composition and/or activity in the gastrointestinal microbiota, thus conferring benefits upon the host's health, have been more limited, and meta-analyses of seven studies using galacto-oligosaccharides and fructo-oligosaccharides provided discordant results, preventing any recommendations [20].

5.2. Toward the "farm pill"—or perhaps the "farm vaccine"

In the last 10 years, parallel to and inspired by epidemiological studies, a number of experimental investigations have been developed in order to characterize those substances and/or organisms that are present in the dust collected from farms and that could be used for allergy prevention. Well-established mouse models of airway allergies have been used to study the anti-allergic properties of these substances and/or organisms, and to get some insight into their mechanisms of action. When inhaled during mice sensitization to ovalbumin, stable dust extract inhibited the development of airway hyper-responsiveness and airway eosinophilia upon challenge, as well as the production of IL-5 by spleen cells and of antigen-specific IgG-1 and IgE. It also suppressed the generation of human dendritic cells *in vitro* [103]. Prolonged exposure to cowshed dust extract reduced the allergy-inducing capacity of dendritic cells through an autocrine IL-10 dependent mechanism [169]. Cowshed dust extracts induced the release of complement factor 5a (C5a), a ligand that has been identified as playing a regulatory role in allergic airway disease; this release was attributed to a serine protease from the midgut of the *Tenebrio molitor* larvae (mealworm), which is present in cowshed dust [170].

Among non-bacterial substances, arabinogalactan—a polysaccharide that is isolated from cowshed dust and plants and is abundant in fodder, particularly in the grass species *Alopecurus pratensis*—was isolated by chromatography and precipitation with specific reagents, and then characterized by nuclear magnetic resonance spectroscopy [171]. Intranasal application of grass arabinogalactan protected mice from developing atopic sensitization, allergic airway inflammation, and airway hyper-reactivity. In addition, treatment of murine dendritic cells with grass arabinogalactan resulted in autocrine IL-10 production, and inhibited their capacity for the induction of an allergic immune response. This allergy-protective effect seems to be specific for grass arabinogalactan; control experiments with gum Arabic- and larch-derived arabinogalactan did not show allergy-protective properties, and structural differences between these forms of arabinogalactan and that sourced from grass were revealed by nuclear magnetic resonance spectroscopy [171]. Anti-allergic and immunoregulatory properties of orally administered *D*-tryptophan from probiotic bacteria were recently disclosed; *D*-tryptophan supplementation also increased intestinal bacterial diversity in mice with allergic airway disease [172]. To our knowledge, these findings have not yet been turned into an application for allergy prevention.

The most extensively studied farm-derived bacterial candidates are *Acinetobacter lwoffii* (*A. lwoffii*) F78, *Lactococcus lactis* (*L. lactis*) G121, and *Bacillus licheniformis* (*B. licheniformis*). These were selected from among more than 100 different bacterial isolates from farms in the ALEX study, based on their relative abundance in cowshed microflora and on the presence of specific serum antibodies in the sera of children living on these farms [173]. *B. licheniformis* spores reduced eosinophilia and goblet cell hyperplasia in the lung tissue of asthmatic mice but provoked an influx of neutrophils that contra-indicated a possible clinical application [174]. Both *A. lwoffii* F78 and *L. lactis* G121 were able to reduce allergic reactions in mice, activate mammalian cells *in vitro*, and induce a Th1-polarizing program in dendritic cells [175]. A positive influence of *A. lwoffii* F78 at the TBet/GATA3 level could be detected, and blocking experiments showed that the molecule responsible was the LPS of this Gram-negative bacterium [176]. Uptake of the Gram-positive *L. lactis* G121 and endosomal acidification were required to stimulate dendritic cells, and signaling via TLR13 appeared to mainly contribute to *L. lactis* G121 cytokine induction in mouse mononuclear cells [177]. Lipoteichoic acids of the *L. lactis* cell membrane are the main candidates for the anti-allergic effect, but their cytokine-inducing activities in human mononuclear cells do not involve TLR2 and TLR4 [178]. The transfer of protection from mothers to their offspring was also demonstrated: The asthma-preventive effect was completely abolished in heterozygous offspring from *A. lwoffii* F78-treated TLR2/3/4/7/9 knockout (KO) homozygous mother mice [179]. *A. lwoffii* F78 inhaled by mothers was shown to act on the offspring at the level of the IFN-γ promoter of $CD4^+$ T cells through an epigenetic mechanism, namely the protection against the loss of histone 4 (H4) acetylation [180].

Staphylococcus sciuri (*S. sciuri*) W620 was selected using a combination of polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) electrophoresis techniques on dust collected from mattresses in farms within the PARSIFAL study [173]. The resulting gel bands of interest, that is, those associated with a specific environmental exposure or disease, were excised and sequenced for determination of the bacterial genus/species. The PCR-SSCP method confirmed the relevance of *A. lwoffii*, the anti-allergic properties of which are described above. It also identified a significant inverse association with childhood asthma for *Lactobacillus* spp. and *Jeotgalicoccus* spp., which is currently under investigation [173]. The protective properties of *S. sciuri* W620 were proven in a Th2-driven ovalbumin model as well as in a mixed Th1/Th2 phenotype house-dust mite model. In the "mixed" model, lymph node cell cytokines of the Th1 and Th2 profiles were decreased in parallel. The activation of the TLR2 and NOD2 receptors, as well as initiation of dendritic cell maturation, followed incubation with *S. sciuri* W620. Dendritic cells that had been exposed to *S. sciuri* W620 selectively supported Th1 cytokine release by co-cultured T cells, despite a lack of IL-12 production due to missing transcription of the IL-12p35 mRNA, and the contribution of IL-23 was shown [181].

The transfer from *in vivo* and mouse experiments to real preventive solutions in humans is an exciting but difficult endeavor. Based

on the above-described preclinical experiments and the somewhat mitigated results of the numerous clinical trials using probiotics, we may present a series of essential points to consider. The first of these points is obviously the difficult transference from *in vitro* results, to *in vivo* experimental results, and then to clinical results in humans. The second point to consider is the route of administration, which could be a key factor in the translation of epidemiological observations into therapeutic/preventive approaches in humans: Preclinical observations in mouse models and the role of dendritic cells in the protective effect should favor intra-nasal administration [173]. However, the impact of the farm-dust-derived bacteria under study on the intestinal microbiota and the effects of their nasal route of administration have not yet been studied. The third point comes from the clear impact of bacterial diversity on the overall effect of the "farm environment," and from the demonstration that each species or strain of organisms exerts its anti-allergic effect through various types of receptors and pathways: Working on mixtures of bacteria and/or their components is attractive, and experimental work is being developed to test their use and to study the possible interactions [173]. However, this work faces several obstacles, including: the production of each component at an industrial scale; the availability and validation of analytical tools to control and ensure both the quality and the stability of every component and of the final mixture; and the increased risk of unwanted side effects, due to interactions between the different components and to interactions of the different components with different cell types in a cascade manner. Such a risk, although acceptable to treat severe diseases, is unacceptable for a prophylactic treatment for healthy children. In addition, if living organisms are used, the risk of a non-pathogen agent becoming a truly harmful pathogenic organism because of a host's immunosuppressed state remains a possibility. The combination of these obstacles immediately raises industrial as well as ethical and regulatory questions [173]. Taking up the challenge of protecting our "farm-deprived" children against allergic diseases in the future should combine the expertise of microbiologists, immunologists, and bioengineers, as well as that of pediatricians, allergists, specialists of clinical trials, and ethical committees.

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