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General

Probiotics under the regulatory microscope

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This review examines current knowledge regarding the safety of probiotic bacteria in man. Tighter and more comprehensive standards and regulations will be developed as probiotic therapy moves from a limited number of products used in the food industry, into more defined therapeutic categories and more complex organisms. A new framework considering probiotics as non-specific promoters of mucosal immunity, defines probiotic characteristics and the clinical circumstances in which it is used. For example, those with immune deficiency taking a high dose of viable bacteria may have an increased risk. A wider range of bacteria is now being used, sometimes in territories other than the gut mucosa. The question of competition with multiple isolates must be addressed, as does the use of nonselected fecal isolates. Transfer of antibiotic resistance with probiotics acting as a 'shuttle' needs clarification. These issues are addressed and reviewed as probiotics evolve into a new therapeutic arena.

Keywords: immunology, *Lactobacillus*, probiotics, toxicology

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1. Introduction

Probiotics as a concept and an entity grew out of the food industry, based on the simplistic notion that has been with us through the 20th century, that certain bacteria are 'good for us'. As the knowledge of the complex microbial systems that relate to the host at mucosal surfaces increases, nothing has substantially changed this view, with < 25% of bacterial species within the gut having been characterised by traditional culture methods [1,2]. In recent years, the knowledge base has expanded in relation to traditional and new probiotic cultures, enabling questions to be levelled at the food industry regarding health targets, characterisation of probiotic isolates, their level of stability and their use in various formulations. At the same time, the concept of what a probiotic is, has changed, primarily due to a recognition that probiotic bacteria can influence physiological outcomes distant from the gut lumen. Moreover, the activation of local mucosal protection mechanisms and the modulation of adaptive immune effector function, can influence levels of protection and degrees of inflammation at all mucosal sites. These observations have led to the idea that some probiotics may best be known as 'immunobiotics' [3]. The concept of probiotics has shifted from a narrow range of dairy isolates that ferment milk to 'promote gut health', to the consideration of a wider range of bacteria that have the capacity to colonise human mucosal surfaces other than the gut, and directly influence pathology at surfaces such as the nasopharynx [4]. Although there is a way to go before probiotic bacteria finalise a place in disease management, the advances made have both increased the number of probiotic isolates and elevated probiotics to be classified as therapeutic compounds, which, in the past, have been the preserve of traditional chemical entities. By moving into the zone of complementary medicines with a database that supports therapeutic claims, and by shifting away from the

traditional probiotic concept, the issue of safety in man requires re-evaluation to comply with the more demanding requirements of biopharmaceutical practice. In this paper, the authors review some of the new directions taken by probiotics in the context of safety requirements expected by the regulatory authorities.

2. The probiotic evolution

In scientific terms, classical probiotics were thought to promote 'gut health' by altering the balance of microbes within the colon, with competitive exclusion of gut pathogens [5]. In separate *in vivo* and *in vitro* studies, probiotic isolates such as *Lactobacillus plantarum* 299V, *Lactobacillus rhamnous* 271 and GG and *Lactobacillus acidophilus* (LAFTI®10), were shown to both colonise the human gut and compete for binding sites on gut epithelial cells [6]. This concept of 'competitive inhibition' of pathogens is microbiologically an attractive mechanism for benefit in infections of the small intestine, in which the total bacterial count is ~ 10^{10} – 10^{11}. In the colon, however, the nature of the indigenous microbiota is different and orally administered probiotics constitute only a very small proportion of the total colonic bacteria count of ~ 10^{14} colony-forming units. This diminishes the argument for competitive inhibition. Recent studies linking clinical benefit in irritable bowel syndrome (IBS) following oral *Bifidobacteria*, with a local and a systemic shift in cytokine secretion patterns, suggest that other mechanisms may operate in the colon [7].

In recent years, clinical studies have focused on mucosal immune enhancement with protection against small bowel infection by rotavirus [8] and on downregulation of allergic hypersensitivity [9]. A review of mechanisms whereby probiotics mediate protection that was published in 2001, concluded that probiotics stimulated cell-mediated immune effector functions, with enhanced secretion of INF-γ by blood cells, enhanced phagocytosis, and an increase in expression of complement receptors on phagocytes [10]. A capacity for circulating cells to secrete an enhanced amount of INF-γ has been demonstrated following cultures with nonphysiological stimuli after the ingestion of probiotics [11,12]. Three observations have progressed understanding of the action of ingested probiotics. First, is the observation that subsets of human dendritic cell precursors express a series of Toll-like receptors, which respond to different microbial antigens [13], and which can discriminate between different presentations of the same microbe [14]. Whole bacteria are required for IL-12 induction, possibly due to a need to activate multiple Toll-like receptors to achieve a skewing of T cell responses towards a T_H1 response [15]. Circulating dendritic cells can be activated to a varying extent by different isolates of *Lactobacillus* species. Activation is detected as upregulation of the maturation of surface markers such as major histocompatibility class II and B7.2, and a secretion pattern characterised by IL-12 and TNF-α. Of importance with respect to a common practice that combines mixtures of probiotic bacteria, was the observation that certain isolates could inhibit the stimulatory effect of others [14]. Combination therapy can be more effective than single species [16], emphasising the importance of selection and assessment using either antigen-processing cells or effector mechanism markers. It is no longer acceptable to mindlessly combine isolates and expect additive outcomes.

Second, a series of studies showed that protection against infection or inflammation due to altered immunity at mucosal sites distant from ingested probiotics, was mediated by a Peyer's patch-driven circulation of T and B cells that preferentially home to the mucosa. This concept became known as the common mucosal system [17]. A murine model of oral candidiasis has been used to study mechanisms of probiotic activity. In this model T_H1 and T_H2 cytokine responses combine to mediate protection against infection [18]. Efficient *Candida albicans* eradication required the integrity of an IL-4 /nitric oxide paracrine loop. This loop is stimulated following ingestion of *L. acidophilus* [19]. Similar outcomes have been described for colonisation of the human female genital tract with pathogens [20]. The number of live probiotic organisms used to achieve these outcomes are of the order of 10^{10} per day – a number similar to the total bacteria content in a healthy small bowel.

Third, oral probiotics can downregulate IgE-mediated allergic hypersensitivity, both in animal models [21] and in atopic infants [9], as a consequence of a skewing of the cytokines towards an INF-γ dominant T_H1 response [21]. This concept fits with the 'hygiene theory' explanation of the high prevalence of allergic disease seen in modern life [22].

Thus, probiotic bacteria may be thought to maximise mucosal protection by driving a T_H1-skewed response through receptor-mediated activation of dendritic cells. This framework enables new methodologies for selecting and assessing probiotic isolates, and asks a different set of questions with respect to safety. Recent studies raise the possibility that much of the 'gut health' noted in the past, involves systemic cytokine-mediated mechanisms [7] rather than a more simplistic competition within the gut for cell-bound receptors.

3. Probiotics – mucosal microbiological therapy

Although much current interest focuses on probiotics driving the 'Peyer's patch – mucosal' immune system at a 'small bowel' level, the origin of probiotic therapy has its roots in the colon as per Metchnikoff's 1907 publication entitled 'On the prolongation of life' where he made 'the recommendation to absorb large quantities of microbes amongst which lactobacilli have an honourable place' [23]. This concept of the 'promotion of gut health' by bacteriotherapy with a direct effect through alteration of colonic colonisation has had a confused history due to poor quality studies with unclear objectives, and the use of ill-defined probiotic species. However, there has now been sufficient studies (often included in meta-analyses) to confirm that particular probiotics can improve clinical outcome in several disorders in which altered colonic microbial colonisation

contribute to clinical disease. Thus, in both children and adults with short-term antibiotic-associated diarrhoea [24,25]. *Enterococcus faecium* SF68 reduced the duration and severity of diarrhoea, and a recent meta-analysis gave strong evidence in support of the value for *Lactobacillus* spp. in reducing the duration of diarrhoea in children [26]. Good quality clinical studies have shown protection following ingestion of a *Lactobacillus* spp. which suppressed the growth of urease-producing bacteria [27], in the context of a day care centre [28].

Interest in control of mucosal inflammation in inflammatory bowel disease (IBD) by probiotics, has been sustained but controversial. In the late 1990s, however, several groups used randomised studies to show that non-pathogenic *Escherichia coli* (in particular *E.coli* Nissle 1917) maintained remission as well as did mesalazine in ulcerative colitis [29,30]. These findings have been confirmed in a more recent double-blind study [31]. The best evidence for a beneficial effect of probiotics in ulcerative colitis came from a study where daily ingestion of a probiotic mixture (VSL#3) that included *lactobacilli, bifidobacteria* and other microbes maintained for nine months was shown to prevent relapse of pouchitis after antibiotic-induced remission [32,33]. Given differences to ulcerative colitis in pathogenesis, caution is needed in extending these observations to colonic disease. The same probiotic mix in an uncontrolled trial not only maintained remission but also induced remission in 75% of patients over 12 months, and reduced the level of inflammation in 87%, all of whom had mild-to-moderate disease [34]. The concept of alteration of an intrinsically pathogenic colonic bacterial flora has been extended to replace colonic bacteria in disease with the rectal infusion of normal commensal faecal bacteria, with excellent results reported in six patients with colitis refractory to standard therapy [35,36]. Evidence for benefit of probiotics in Crohn's disease is less clear, with no benefit following *Lactobacillus* GG [37], but an apparent prevention of relapse with *E.coli* Nissle [38] and some benefit in a group of paediatric patients undergoing steroid withdrawal [39].

The most controversial area in assessing 'gut health' using probiotics has been in IBS, due to the heterogenicity of disease, subjective end points and poor quality trials using multiple badly characterised probiotic isolates. This has led to calculations that suggest a study of up to 300 subjects is needed to obtain significant results. Many reviews confirm this unsatisfactory position though recent emphasis on more narrow clinical classification, the use of better quality products and clearly defined end points, and better experimental design, has led to significant outcomes with lower numbers (e.g., 7). Part of the problem is the lack of a mechanistic framework to understand gut dysfunction in this common disorder, though disturbed colonic colonisation, often following antibiotic therapy or environmental shifts, has become increasingly accepted [40]. The acceptance, in part, follows on increasing database from placebo-controlled studies using individual probiotic bacteria or a limited mix of bacterial species [41,42] showing clinical benefit, especially in relieving symptoms such as pain and bloating due to gas accumulation. A recent Irish study is particularly helpful. IBS patients given *Bifidobacteria infantis* had lower symptom scores than those taking *Lactobacillus salivarius* or placebo. In addition, the IL-10/IL-12 ratio was less than that in matched controls (a result consistent with findings previously described for colonic mucosal biopsies by this group). The reduced value for the IL-10/IL-12 ratio in blood mononuclear cells increased following *B.infantis* but remained reduced in the *L.salivarius* and placebo groups [7]. The importance of this study is that it underlines the value of some but not all bacteria, it identifies a metabolic abnormality that may lead to a better understanding of the pathogenesis and classification of subgroups of subjects with IBS, and gives a surrogate marker to assess clinical benefit in future clinical trials. In addition, colonisation with normal gut flora has been shown to improve gut function [43]. Perhaps the most dramatic clinical improvement following recolonisation of the colon with faecal infusions has been in subjects with acute and chronic *Clostridium difficile* infection [44-45]; many of these subjects present with diarrhoea-predominant IBS.

Where does this leave us with respect to probiotics and colonic disease? First, it is not clear whether or not a selected single species of probiotic is as effective as multiple isolates or nonselected faecal bacterial infusions from normal donors. It is unclear whether some subjects may benefit from one preparation with others responding to a different probiotic. The recent use of cytokine ratios measured from colonic biopsies or blood culture is an exciting step forward, though additional markers are needed and will be found. The specificity of isolate for an outcome in IBD is equally confusing, though mixtures and colonic fecal infusions may be of value until more reliable commercially available isolates become available. The use of a wide range of bacteria, however, including 'normal colonic bacteria', raises additional safety issues that must be addressed, in the selection of isolates, the screening of sources, and the monitoring of recipients [43].

4. Probiotics – safety issues

Traditionally, probiotic cultures have been delivered to the gut as dairy products or in a lyophilised from in capsules or tablets. The active principal was defined in terms of 'live bacteria at the time of production' – with little or no attempt at quality control. Recently, probiotics have been included in foods other than diary including infant formulae, cereal-based products and fruit juices. Many new probiotic cultures as well as methods and sites of administration, have also been introduced in recent years as the range of clinical targets has increased. Consequently, since the early acceptance of a high safety margin due to protracted human use of a restricted number of probiotic isolates, there is now a wider range of delivery vehicles and many new isolates and mixes in current use, which have been less scrutinised and sometimes less characterised. With better production and quality control, viable bacteria are present in much greater numbers than was found

in early food products, raising additional safety issues related to dose.

To date, most probiotic cultures used have been bifidobacteria and lactic acid bacteria (LAB) [46-47]. More recently, new probiotics have been introduced, including spore-forming Gram-positive bacteria [48], yeast [49] and *E. coli* [50], as well as *Bacteroides* spp. and undefined mixtures, including fecal infusion [43,51-52]. The use of live endospore-forming bacteria, such as *Bacillus* spp., as probiotics is not GRAS (generally regarded as safe). Some isolates from this group, including *B. cereus*, are known to produce enterotoxins [53]. Such endospore-forming bacteria are used as probiotics in many countries, despite reports on infection following their consumption [54-56]. *E. coli* have been used as probiotics [50], notwithstanding that there are many pathogenic strains of this species.

Though traditional isolates of LAB are GRAS and would normally be considered safe, there have been increased efforts to ensure that is the case for clinical use. With the development of new LAB strains and using improved manufacturing, the consumer is exposed to high concentrations of live microorganisms, with each capsule containing numbers of live bacteria similar to the total bacteria count in the normal small bowel. Importantly, as the immune-modulating effect of many probiotic bacteria becomes recognised, vulnerable subgroups such as the elderly and/or immunocompromised people will be given these products.

Three areas of potential toxicity have been addressed with respect to all probiotics. The first is their potential for transmigration. Occasional cases of endocarditis due to *Lactobacilli* have been reported [57-59] and an early report suggested that clinical isolates may be particularly adhesive to platelets [59]. However, there is no clear evidence that clinical isolates are more adhesive than other related *Lactobacilli* isolates [59]. In clinical practice, however, such cases are very rare [60] and a recent review in Scandinavia failed to find any correlation between the incidence of bacteremia and clinical usage of probiotic *Lactobacilli* [61]. This latter study showed that in most cases with a positive blood culture for lactobacilli, other bacteria were also detected [61]. Although the safety of long-used probiotic isolates with respect to translocation has been accepted, these rare reports alert the need for continued surveillance, especially in those subjects with impaired mucosal immunity. Finally, it should be noted that, paradoxically, some probiotics may reduce translocation of coliforms as a result of enhanced mucosal immunity and an associated reduced transmucosal uptake of enteric bacteria (unpublished results).

Second, as for all microorganisms, probiotics have a range of physiological and metabolic characteristics that may impact on the physiology, immunology and microbiology of the gastrointestinal tract. Also, probiotic bacteria may produce metabolites that can be undesirable under particular conditions. For example, short small bowel (SSB) syndrome is an uncommon condition where certain lactobacillus may have undesirable effects [62,63]. In SSB, lactobacillus may reach levels of up to 10^{12} CFU per gram of faeces [63,64]. At these levels, certain metabolites have a negative impact on the host. Deconjugated bile acid is one such metabolite. This metabolite is formed on hydrolysis of glycine or taurine-conjugated bile acids into the amino acid residue and free bile acid, a reaction catalysed by bacteria-derived bile salt hydrolase. Under normal conditions, conjugated bile acid is reabsorbed in the ileum together with lipid and lipid-soluble vitamins. If bile acids are deconjugated by lactobacilli, little is reabsorbed and, thus, significant amounts of deconjugated bile enters the colon together with non-absorbed lipids and vitamins, leading to malnutrition. Unabsorbed bile acid may also increase the risk of colon cancer [65]. D-lactate is another metabolic product relevant to SSB syndrome. Acidaemia and aciduria is seen due to high intestinal levels of D-lactate produced by certain lactobacilli [64,66]. *L. acidophilus* (and related species) are commonly found in faeces in SSB syndrome [64]. The ratio of D to L lactate in faeces of these patients is 60:40%, which is similar to that found with some commercially available isolates such as *Lactobacillus johnsonii* [66]. Coliforms used as probiotics may have specific toxicity due to a release of LPS. Some strains of *E. coli* are β-haemolytic, as are strains of enterococci. β-Haemolytic bacteria should not be used as probiotics, and an absence of haemolytic activity should be confirmed before using new isolates of potentially haemolytic strains of bacteria as probiotics. Gram-positive bacteria do not contain LPS.

Third, the question of antibiotic resistance is important. First, it is relevant to antibiotic therapy in the rare instance of infection. Second, there is a concern related to transmission of antimicrobial resistance from probiotic cultures to pathogens, due to a shuttling of resistance genes particularly from enterococci. Transferable antibiotic resistance is, of course, relevant to use of antibiotics in general. Indiscriminate use of antibiotics in human and veterinary medicine, agriculture and aquaculture, over time, has caused an increase in resistant variants among both pathogen and commensal organisms [67]. The abuse of antibiotics promotes spread of acquired resistance to different microbial populations. There is no obvious barrier to the transfer of genetic material between pathogens, potential pathogens and commensal LAB, and this could lead to acquired assistance [68]. Thus, a wide exchange and expression of the antibiotic resistance determinants, between microorganisms of various taxonomic groups, is likely to take place. The mammalian gastrointestinal tract provides favourable conditions for the transfer of genetic material between many species of bacteria [69]. Resistance genes could be transferred not only between members of the resident gut flora, but also to and from transient probiotic flora. Much more information is needed on whether or not probiotic bacteria can facilitate the spread of antibiotic resistance in such circumstances. Current negative data with respect to antibiotic/gene transfer with respect to probiotic bacteria are welcome [70-73], but caution must be used in extending these observations to different probiotic organisms and different circumstances.

Issues of safety have become more focused due to recognition of a mechanistic framework within which probiotics act and recognition that defined and high concentration viable products will become available as evidence-based complementary medicines. The concept of 'probiotic' has shifted to include the therapeutic use of microbes other than lactobacilli and bifidobacteria, as well as the application of probiotic therapy via nonoral routes. As the science and clinical relevance of probiotics advances, so too does the nature of the subject who will take probiotics as part of their therapeutic regimen. Subjects with defective immunity, abnormal gut structure and others with metabolic defects, may handle probiotics in a different fashion to those with healthy individuals who, in the past, used 'classic' probiotics 'to promote health'. Safety in terms of the immunomodulatory effect of probiotic bacteria must also be considered (as subtle shifts of cytokine balance may lead to unexpected clinical outcomes).

As a baseline, it is useful to look at guidelines developed by the food industry. In 2002, guidelines for the evaluation of probiotics in food were drafted by a joint Futures and Options Association/WHO working group [74]. This group proposed that strains to be used as probiotics should be characterised at a minimum with the following tests:

- Determination of antibiotic resistance patterns. A selection of antibiotics to be tested has also been proposed [75];
- Assessment of certain metabolic activities (e.g., D-lactate production and bile salt deconjugation);
- Assessment of side effects during human studies;
- Epidemiological surveillance of adverse effects in consumers (postmarket);
- If a strain belongs to a species that is a known mammalian toxin producer, it must be tested for toxin production;
- If the strain under evaluation belongs to a species with known haemolytic potential, determination of haemolytic activity is required.

Given the changes in probiotic usage discussed above, additional criteria need to be addressed. This will be an ongoing process as more information becomes available, but, at this time, these criteria would include:

- Infectivity in immunocompromised animals with high dosage of viable organisms;
- Immunoregulatory shifts due to a change in cytokine balance (suggest: autoantibody screen; quantitative intradermal skin tests with a standard batch of allergens; immunoglobulin levels including IgG subclass);
- Mixtures of probiotics need to be assessed in *in vitro* culture to avoid inclusion of isolates that can block the cytokine stimulation of another isolate [14];
- Donor screening, to ensure no transfer of pathogens (especially where multiple isolates are planned for recolonisation of gut or other mucosal sites).

More extensive observation in man pre and postcommercialisation of new isolates must occur. Particular attention to high-risk subjects is needed, as they will increasingly be the recipients of the probiotic in question. Examples of such people are immune compromised subjects, those with short small gut, and those at the extremes of age. This list will expand as evidence accumulates to support the use of a wide range of probiotic bacteria for therapy in new areas of human disease.

5. Expert opinion

The aim of this article was to review the toxicology of probiotics. From the start, it was clear that a laxity of rigor in awareness of potential problems was creeping in, based on a long-term use of certain lactobacillus species and bifidobacteria in fermented foods, where essentially no untoward effects had been noted. What had not been considered was that probiotics were seen as a 'food' taken by normal individuals looking for 'health promotion'. Despite claims to the contrary, the numbers of viable probiotic bacteria in these products were low – many logs less than the preparations now coming to market defined as complementary medicines. The idea that lactobacillus species could be pathogenic, however, was highlighted by the occasional case report of bacterial endocarditis, though such cases have not been linked to the use of probiotics, and reports of local metabolic abnormalities in some subjects with abnormal gut structure.

In the last few years a seachange has come over probiotic usage, to the extent that a name change has been suggested ('immunobiotics'). For the first time mechanisms of action are being understood and good clinical studies are being performed (unfortunately not contemporaneously), moving probiotic usage into the clinical arena. This has meant:

- That the probiotic product has been better defined in terms of component, formulation, viability, dosage and presentation. Within this new framework, new 'probiotic' bacteria are being used to attain particular outcomes, even Gram-negative bacteria which have their own potential for toxicity. Evidence suggesting dosage of around 10^{10} viable bacteria is needed, and the development of improved fermentation and formulation techniques, has led to potent high-dose preparations appearing on the market. For 'classical' probiotic bacteria, such as *L. acidophilus*, there have been no reports of toxicity but as formulation now aims at release of these bacteria within the small bowel to activate Peyer's patches, long-term assessment of these altered conditions becomes important. These numbers of viable bacteria are similar to the total bacteria count within the normal small bowel. For 'newer' organisms, such as *E.coli*, chosen as probiotics to be used in particular clinical situations, a new safety platform has to be established as some of these bacteria have the potential to secrete toxins, or invade tissues. Where outcomes are desired at particular mucosal sites such as the nasopharynx or the colon, direct application of probiotics may cause local or distant toxicity due to metabolic changes or immune crossreactivity.

- That a different group of subjects are taking probiotics, identified by clinical need. Thus, subjects at the extremes of age, immune deficiency, or mucosal disease, will be taking a range of probiotics. Therefore, it is important to ensure safety in subjects with structural and immune disorders, who may be prone to particular toxic outcomes.
- With better definition of mechanism, a new range of undesirable outcomes of probiotic therapy must be considered. For example, competition for Toll-like receptors on antigen-presenting cells (with colonising bacteria, or within mixes of probiotics) must be assessed, as it is possible that impaired immune responses may occur with some probiotics or with some mixtures. Further, switching the balance of T cell cytokine secretion, although desirable with respect to allergic disease, may be a cause for concern in other circumstances, such as subjects with autoimmune disease, or a family history of autoimmunity. In this case, a drive to secrete INF-γ may actually promote autoimmunity. Safety screens should include autoantibodies with titres.

These comments are not meant to dampen current enthusiasm about probiotic usage, but rather to point to a set of quite different circumstances and frameworks, in which these bacteria are now being used in man. The move from 'food' to 'complementary medicines', brings probiotics under a new regulatory microscope. The importance of assessing probiotic toxicology in a completely new context – dosage, formulations, bacterial species and target populations – is stressed. The question of undesirable outcomes of altered immunoregulation will require long-term clinical observation. The lessons of serious infections and autoimmune disease occurring in subjects with rheumatoid disease and Crohn's disease treated with monoclonal antibodies targeting TNF-α leading to a disturbance of the immune regulatory network, must not be forgotten.

Finally, and of great importance as it may affect the wider population, is the extent to which probiotics in the gut can become a shuttle mechanism for transfer of antibiotic resistance genes. There is some urgency for both laboratory and epidemiological studies to be set up to address this important question.

Who is to be responsible for the assessment of safety issues? To date, little has been done, with most comment coming from committees related to the food industry. Clearly this is inadequate for the 'new probiotic' era, and government regulatory authorities will need to develop new sets of guidelines for both probiotic manufacturers and scientific bodies to assess. There is also likely to be a major shift in probiotic production. Currently, many small producers provide numerous poor quality products with little or no quality control. Viability is low, they often contain untested mixes of bacteria and have no proven value. The regulatory demands that will be made for the products of the future will require resources that are beyond those of most of these producers, as well as GMP production facilities relevant to human medical consumption. The future is clearly with major biopharmaceutical enterprises who have the resources to develop this new and exciting area of therapy.

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- **A start regarding guidelines, but traditional approach needs to be modernised.**

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