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Probiotics revisited: new strains, new benefits, new opportunities

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INTRODUCTION

Probiotic bacteria have become an established part of the functional food market, and are being found in a growing number of food formats with a growing number of associated health benefits. Yet challenges remain, particularly around maintaining the high consumer confidence in probiotic bacteria. Poorly characterised probiotic strains with poorly substantiated health claims mean that probiotics could lose credibility despite the wealth of evidence that specific probiotic strains can and do exert demonstrable health benefits. Thus, it is imperative that each probiotic bacterial strain is supported by clinically-relevant and peer-reviewed research.

Fonterra is the world's leading exporter of dairy products, responsible for around one third of the global dairy trade. Based in New Zealand, Fonterra is a co-operative company owned by more than 12,000 dairy farmers, and exports a wide range of dairy-based commodity and specialty products to over 140 countries. In the mid 1990's, Fonterra embarked on a program to develop proprietary probiotic strains that targeted gut health and immune enhancement benefits. Based on «classical» selection criteria, including acid and bile tolerance (to mimic conditions encountered in the human gastrointestinal tract), initial safety determination, and immune-modulating activity, a starting pool of 2000 lactic acid bacterial strains were reduced to 4 candidate probiotic bacterial strains, [1]. Of these *Lactobacillus rhamnosus* HN001 (trademarked as DR20) and *Bifidobacterium animalis* subspecies lactis HN019 (trademarked as DR10) were eventually commercialised. The on-going probiotic research and development program at Fonterra is underpinned by the FAO/WHO definition of probiotic bacteria as «live microorganisms that, when administered in adequate amounts, confer a health benefit on the host» (FAO/WHO Report, 2001), [2], in that an ideal probiotic bacterial strain must be safe, provide a demonstrable health benefit, and reach the gastrointestinal tract of the consumer in a live state.

PROBIOTIC SAFETY

Safety is paramount for a functional food ingredient. While both drugs and functional foods, including probiotics, must by definition provide some kind of benefit to the consumer, the uncontrolled nature of functional food consumption means that probiotic bacteria must be shown to provide no risk to human health even when consumed in high doses. Both DR10 and DR20 have been the subject of extensive safety testing, from *in vitro* studies through

to randomized, double-blind placebo-controlled human clinical trials. Animal trials have consistently shown that DR10 and DR20 consumption were not associated with adverse effects on haematological, histological, and growth parameters [3–5], even at high doses, and did not show significant translocation across the gut barrier. It was also established that neither strain exacerbated the progression of an immune disease in a mouse model of experimental autoimmune thyroiditis [6], an important finding given the immune-modulating activity of the strains. Furthermore, *in vitro* analysis revealed that neither DR10 nor DR20 could degrade intestinal mucin or led to platelet aggregation or activation [7–8]. Both strains exhibited antibiotic resistance profiles typical of their respective species [9]. Genome sequencing of both strains found no evidence of transferable antibiotic resistance genes, or indeed any other genes commonly associated with obvious virulence or pathogenicity. Therefore, the *in vitro* and *in vivo* safety studies have consistently shown that DR10 and DR20 can be considered as non-pathogenic microorganisms and are safe for human consumption.

PROBIOTIC EFFICACY

Another important feature of probiotics is their ability to colonise, if only transiently, the human gastrointestinal tract. Human dietary intervention studies using DR10 and DR20 have shown that both strains can be recovered from the faeces, indicating that the strains can survive passage through the human gut, but do not appear to lead to permanent colonisation [10–13]. An interesting finding from the human studies was that the probiotics appeared to have prebiotic-like effects, in that both strains increased the levels of other «good» bacteria (lactobacilli and bifidobacteria). For instance, one feeding trial showed that prior to the addition of DR20 to the diet, some trial participants exhibited unstable lactobacilli populations, which were then stabilised by the presence of DR20 [10].

The Fonterra probiotic strains may also exert other benefits to the human microflora as several animal trials have provided evidence for anti-pathogen effects [14–18]. For instance, mice fed DR20 and then exposed to pathogenic *E. coli* strain O157:H7 showed reduced morbidity, reduced bacterial translocation, and increased markers of innate and acquired immunity compared to mice fed the pathogen only, and DR20 was able to almost completely protect mice from death due to *Salmonella typhimurium* infection [14]. Similarly, DR10 consumption protected weaning piglets

from rotavirus- or *E. coli*-associated diarrhoea associated with rotavirus or infection [16], and greatly reduced the pathogenic effects of *E. coli* O157:H7 and against *S. typhimurium* in mice [17]. While anti-pathogen effects in human subjects is more difficult to study, a recent study that provided DR20 along with *Lactobacillus acidophilus* to elderly subjects in a cheese format showed that the probiotic strains were able to reduce intestinal colonisation by the pathogen *Clostridium difficile*, an opportunistic pathogen and leading cause of antibiotic-associated diarrhoea [19].

Immune protection is seen as another important component of probiotic health benefits. Two important markers of immunity, phagocyte activity and natural killer (NK) cell function, are concerned with immune surveillance, and have been extensively studied for DR10 and DR20. Various cell types, such as neutrophils, dendritic cells and macrophages, are able to engulf particulate matter such as invading pathogenic bacteria, and can alert the rest of the immune system to the presence of infectious agents or other threats [20]. In contrast, NK cells target other host cells, with their ability to recognise and kill virally-infected or tumour cells [21]. Hence both activities play vital roles in disease protection. The ability of both DR10 and DR20 to stimulate the function of both phagocytes and NK cells has been consistently shown across multiple animal studies and human feeding trials [17; 20–29]. In addition to the benefits of DR10 and DR20 on innate immunity, there is also evidence that the probiotic strains can enhance aspects of adaptive immunity, with beneficial effects on antibody responses [25]. It is interesting to note that the apparent immune effects of DR10 and DR20 are due to increased immune cell activity rather than proliferation [4–5], which suggests that the strains do not exert strong pro-inflammatory effects, as supported by some findings from human feeding trials [19].

DR10 and DR20 have both shown efficacy in gut health outcomes. In a recent study of probiotics and intestinal barrier function, DR20 was the strongest performing probiotic from a panel of common commercial strains in an *in vitro* assay of gut barrier integrity [30]. There is an increasing awareness of the importance of the gut barrier function to health, and disruption to this barrier appears to have a range of detrimental effects [31–32]. Another aspect of gut health is colonic transit time. Slow transit times have been associated with irritable bowel syndrome (IBS), constipation and increased colonic cancer risk [33–35]. A recent double blind, placebo-controlled study involving DR10 in human adults has shown that DR10 can improve colonic transit time in a dose-dependent manner [36]. In addition, DR10 consumption was associated with significant improvement in self-reported gastrointestinal symptoms such as regurgitation, abdominal pain, nausea, constipation, and irregular bowel movements. These findings have important health implications as constipation and gut discomfort are common symptoms in the general population. The findings of the DR10 CTT trial are currently being confirmed in repeat human clinical trials.

The use of probiotics for paediatric applications is becoming increasingly common. This is supported by the health benefits and safety record established in adult populations, and also by the growing number of reports of lactobacilli and bifidobacteria being present in human breast milk [37–38]. This, along with studies of the infant microflora, suggests that bifidobacteria, as well as lactobacilli, are natural components of the infant microflora, and may play important roles in the neonatal gastrointestinal tract and immune system [39–40]. Even so, given the relative vulnerability

of paediatric populations, there needs to be a compelling reason to include probiotics as a normal part of the infant diet. While both DR10 and DR20 have shown consistent benefits in terms of pathogen protection, enhanced immune protection, and improved gut function and/or gut comfort in adult human populations, tangible health benefits must still be shown in paediatric populations.

DR10 (BIFIDOBACTERIUM ANIMALIS SUBSPECIES LACTIS HN019) HUMAN CLINICAL STUDIES

DR10 was the subject of a large randomised, double blind, and placebo-controlled study to examine the impact of DR10 and the prebiotic galacto-oligosaccharide (GOS) on measures of paediatric morbidity [41–42]. The trial involved two groups of 312 health children aged 1 to 3 years. One group received milk fortified with vitamins and minerals, while the other received the same fortified milk with added DR10 and GOS. The children received the treatments twice daily for 12 months. At the end of the 2-year follow-up period, the group fed the fortified milk with the added DR10 and GOS experienced a number of significant health benefits compared to the control group, with reduced disease risks across a range of childhood morbidities including dysentery (21%) (95% CI: 0 to 38%; $p = 0.05$), pneumonia (24%) (95% CI: 0 to 42%; $p = 0.05$), and severe-acute lower respiratory infections (35%) (95% CI: 0 to 58%; $p = 0.05$). This led to an overall 16% (95% CI: 5 to 26%, $p = 0.004$) drop in days with severe illness, a 5% (95% CI: 0 to 10%; $p = 0.05$) drop in febrile illness, and a 6% (95% CI: 3 to 9%; $p < 0.001$) in antibiotic use associated with the use of DR10 and GOS. In addition to the measures of paediatric health, the study also revealed that DR10 may also influence iron status, with the DR10 and GOS group showing a 45% (95% CI 11%, 66%; $p = 0.01$) reduction in the risk of anaemia and iron deficiency. Taken together, it appears that DR10 consumption can lead to several health benefits based on both improvements in gut function and immune function. While further research is required to confirm some of these findings, and to explore the underlying mechanisms of DR10's probiotic effects, there is still clear evidence of meaningful efficacy in both human adult and paediatric settings.

DR20 (LACTOBACILLUS RHAMNOSUS HN001) HUMAN CLINICAL STUDIES

While DR10 has shown very promising efficacy in gut health, DR20 has emerged as a leading probiotic strain in the treatment or prevention of paediatric allergies, especially eczema (also known as atopic dermatitis). The anti-allergy efficacy of DR20 is based mainly on a double-blind, randomized, placebo-controlled trial that studied the role of DR10 and DR20 on infant allergy, conducted in New Zealand [43–45]. While the rates of childhood allergy have been increasing in many countries worldwide, the rates appear to be particularly high in NZ. Indeed, the incidence of eczema in 6–7 year olds measured as part of the international ISAAC study, was reported to be 27% [46–47]. The probiotic study was designed to assess eczema symptoms and severity in infants at risk of allergy (i.e. one or both parents having had a physician-diagnosed allergic disease in some point in their lives) over the first 2-years of life, and then to assess the later development of allergic disease via two follow-ups as the infants turned 4 and 6 years of age. Approximately 450 pregnant mothers were randomized to one of three groups, and received daily doses of either DR10 (9×10^9 CFU/day), DR20 (6×10^9 CFU/day), or placebo from 35 weeks gestation until birth, and for up to 6 months after birth if breastfeeding. The infants then

received the same treatment as their mother as a daily supplement from birth until 2 years of age.

For the initial 2-year study [44], eczema prevalence and severity were assessed at 3, 6, 12, 18, and 24 months according to strict diagnostic criteria, while eczema severity was assessed using the SCORAD (SCORing Atopic Dermatitis) tool. To assess atopic status, skin prick tests performed were performed on the infants at 2 years of age using common allergens (egg white, cat pelt, house dust mite, mixed grasses, peanut and cow's milk). Results of the trial revealed a dramatic drop in both eczema prevalence and severity associated with consumption of DR20. Of the infants that received DR20, only 21 out of 144 (14.6%) were diagnosed with eczema at 2 years compared with 37 out of 152 (24.3%) in the DR10 group and 40/150 (26.7%) in the placebo group. This led to an odds ratio of developing eczema in the DR20 group of 0.47 (95% CI 0.26–0.84, $p = 0.01$), and a number needed to treat of just 8.3. Interestingly, a beneficial effect for DR20 was also observed for eczema severity, with a significant reduction in the risk of developing a SOCRAD of at least 10 (indicating at least a mild-to-moderate level of eczema symptoms) in the DR20 group (22.9% DR20 vs. 38.7% placebo, OR = 0.47; 95% CI = 0.28–0.78) but unchanged in the DR10 group. Rates of atopic sensitisation to specific allergens as indicated by skin prick tests were not significantly different between the three groups, and proportional drop of eczema prevalence between the placebo and DR20 groups was similar between IgE- and non-IgE-associated eczema, which suggests that DR20 may act by reducing the allergic response rather than allergic sensitisation. Given this hypothesis, it was interesting to speculate whether the reduced eczema effect of DR20 could be maintained beyond the end of the treatment period, that is, whether the eczema rates in the DR20-treated group would increase back up to placebo levels after the cessation of DR20 feeding. However, results of the 4-year follow-up study clearly showed that the anti-eczema efficacy of DR20 was maintained even though the study participants had not consumed DR20 for 2 years. In the DR20 group, 27.2% (37 of 136) of children had eczema symptoms compared to 39.2% (56 of 143) of children in the placebo group (OR = 0.58; 95% CI = 0.35–0.96, $p = 0.04$). While the difference in the proportion of children with SCORAD ≥ 10 between the DR20 and placebo groups was no longer statistically significant, a clear trend for reduced symptom score in the DR20 group was still evident.

As shown by several recent meta-analyses [48–49], few probiotic studies have shown significant anti-eczema efficacy, and even fewer studies have followed their respective study populations to examine the long term consequences of probiotic intake as infants on allergy development in childhood. A landmark study in this regard is the *Lactobacillus rhamnosus* GG (LGG) study, which published data as the study participants reached 2, 4, and 7 years of age [50–52]. While LGG showed an initial benefit on eczema prevalence at 2 years, which was maintained at 4 and 7 years, there was a strong trend for increased rates of rhinitis and asthma in the LGG-treated group compared to controls [50–51]. While these rates did not reach statistical significance, the results are still a cause for some concern. In the case of DR20, an important part of the 4-year follow-up was the assessment of allergic disease other than eczema. The findings indicated that DR20 consumption appeared to have no affect on the

prevalence of asthma or wheeze, and resulted in reduced prevalence of rhinitis (37.5% drop in the DR20 group compared to the placebo), hay fever (42.4% drop) and rhinoconjunctivitis (61.6% drop) (results in press).

While the second follow-up study as the study participants turn 6-years of age will re-examine the rates of allergic disease, the results obtained at 2 and 4 years suggest the possibility that the benefits of DR20 for eczema prevention may extend to allergic disease in general. This hypothesis is backed up by at least three lines of evidence. First, as part of the eczema prevention trial, samples of breast milk and cord blood were taken from a subset of participants. Cord blood samples from the DR20 group had higher levels of interferon γ than cord blood from the placebo ($p = 0.03$) [53]. This is significant as neonatal interferon- γ levels are associated with reduced allergy risk [54]. Second, a previous eczema treatment trial that fed a combination of DR10 and DR20 or a placebo to groups of children with pre-existing eczema showed an enhanced rate of eczema resolution for the DR10 and DR20 group, at least in the subset of children sensitized to food allergens [55]. This suggests that DR20 may have efficacy for both eczema prevention and treatment, but this will need to be supported by future human clinical trials. Third, a recent study in a pig model of allergic disease showed that DR20 treatment effectively reduced the severity of both asthma and eczema symptoms [56].

The extension of the anti-eczema benefit of DR20 beyond the initial feeding period did not appear to be due to the permanent colonisation of the infant microbiota by DR20. While there was evidence of enhanced *Lb. rhamnosus* colonisation in the DR20-ed group during the treatment period, the rates of colonisation by DR20-like *Lb. rhamnosus* strains were between the three treatment groups at 4 years.

CONCLUSIONS

A feature of the paediatric eczema study involving DR10 and DR20 was the strong focus on probiotic safety. Consumption of significant amounts of either probiotic daily from birth until 2-years of age was not associated with any increase in reported adverse events compared to the placebo group, and did not affect measures of growth even up to 4-years of age. These findings are important as the detailed analysis of probiotic safety in paediatric populations is comparatively rare. Partly based on these results, DR20 has been recognised as having «generally regarded as safe» (GRAS) status by the United States Food and Drug Administration (USFDA) for use in both adult and infant food applications.

In conclusion, the probiotic nature of DR10 and DR20 are supported by significant studies examining both the efficacy and safety of these strains. In addition, significant work has also been carried out investigating the stability of the strains in various food formats [22; 57]. While both DR10 and DR20 have shown evidence for immune protection and anti-pathogen benefits, human clinical trials have shown efficacy for DR10 for reduced constipation through improved colonic transit time in adults, and reduced infectious disease rates in children, and efficacy for DR20 in preventing allergy. It is clear from these and other studies that all probiotic strains are not equal, and that specific probiotic strains have specific benefits. On-going studies are continuing to confirm and extend the known benefits of the DR10 and DR20 probiotic strains.