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Review article

Monostrain, multistrain and multispecies probiotics —A comparison of functionality and efficacy☆

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Abstract

This literature review was carried out to make a comparison of functionality and efficacy between monostrain, multistrain and multispecies probiotics. A monostrain probiotic is defined as containing one strain of a certain species and consequently multistrain probiotics contain more than one strain of the same species or, at least of the same genus. Arbitrarily, the term multispecies probiotics is used for preparations containing strains that belong to one or preferentially more genera. Multispecies probiotics were superior in treating antibiotic-associated diarrhea in children. Growth performance and particularly mortality in broilers could be improved with multistrain probiotics. Mice were better protected against *S.* Typhimurium infection with a multistrain probiotic. A multispecies probiotic provided the best clearance of *E. coli* O157:H7 from lambs. Rats challenged with *S.* Enteritidis showed best post-challenge weight gains when treated with a multispecies probiotic. Possible mechanisms underlying the enhanced effects of probiotic mixtures are discussed. It is also emphasized that strains used in multistrain and multispecies probiotics should be compatible or, preferably, synergistic. The design and use of multistrain and multispecies probiotics should be encouraged.

Keywords: Probiotic; Lactic acid bacteria; Efficacy; Monostrain; Multistrain; Multispecies; Microbial interaction; Synergism; Symbiosis

Contents

Abbreviations: B., *Bifidobacterium*; *Ec.*, *Enterococcus*; LAB, lactic acid bacteria; *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *S.*, *Salmonella*; *St.*, *Streptococcus*.

☆ Taxonomic names are used according to the present nomenclature as published by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany (DSMZ; www.dsmz.de).

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1. Introduction

There is ample evidence from laboratory experiments that ingestion of probiotic microbes, especially lactic acid bacteria and bifidobacteria, alleviates or prevents various disorders, such as lactose intolerance, rotavirus diarrhoea and atopy (Ouwehand et al., 2002a). Despite this evidence, functionality of the probiotics in practice remains questionable. The main reason may be that commercial probiotic products often do not meet a quality standard in that composition and viability are variable (Fasoli et al., 2003; Hamilton-Miller and Shah, 2002; Hamilton-Miller et al., 1999; Temmerman et al., 2003; Weese, 2002). A second major issue in relation to the application of probiotics is the poor evidence for efficacy as based on clinical trials (Klaenhammer and Kullen, 1999).

There are at least three issues that interfere with the identification of specific health effects of probiotics (Klaenhammer and Kullen, 1999). First, the complexity and variability of the gastrointestinal environment in relation to gastrointestinal diseases complicate the description of clear effects of probiotics on health and disease. Secondly, the confusion as to identity, viability and activity of probiotic strains contributes to the misidentification of cultures used in clinical investigations. Thirdly, single probiotic strains (monostrain probiotics) are assumed to induce a multitude of effects among different individuals in a test population.

Functionality of a multistrain probiotic could be more effective and more consistent than that of a monostrain probiotic. Colonization of an ecosystem providing a niche for more than 400 species in combination with individually determined host-factors is anticipated to be more successful with multistrain (multispecies) probiotics than with monostrain preparations. Indeed, Famularo et al. (1999) have envisaged that probiotic preparations containing bacteria of only one strain have little chances of successfully colonizing the GI-tract. Furthermore, probiotics are expected to control multi-factorial diseases demanding a variety of probiotic properties, whereas such properties are strain-specific (Sanders and Huis in't Veld, 1999). Therefore Dunne et al. (1999) and Rolfe (2000) have suggested that probiotics should consist of a combination of strains. In 1992 a group of probiotic experts concluded that the optimal prophylactic culture is a mixed one: 'Different strains can be targeted toward different ailments and can be blended into one preparation' (Sanders, 1993). Mixed cultures may contain bacteria that complement each other's health effect and thus have synergistic probiotic properties.

Furthermore, research with probiotic strains aims at unraveling mechanisms of action which can be claimed for one specific strain. The elucidation of underlying mechanisms for multistrain probiotics requires sophisticated study designs that are expensive (Klaenhammer and Kullen, 1999). A further drawback is that most clinical studies are funded by companies with interest in one specific strain only (Sanders and Huis in't Veld, 1999). Finding a single strain with unique properties can lead to patents whereas the clinical effectiveness of multistrain probiotics is not easily patentable.

The aim of this review is to compare the efficacy of multistrain and multispecies probiotics with that of monostrain probiotics. We have been able to identify only a limited number of publications explicitly dealing with this topic, but much more valuable information could be obtained from other publications. In these studies animals or humans with a normal gastrointestinal flora were administered different types of probiotics of the lactic acid bacteria genera. It is important to stress that most studies were not designed to compare the efficacy of multi- versus monostrain

probiotics. For the purpose of this review we have created a new set of probiotic definitions regarding their strain composition. Monostrain probiotics are defined as probiotics containing one strain of a certain species, and consequently multistrain probiotics contain more than one strain of the same species or closely related species, for instance *Lactobacillus acidophilus* and *Lactobacillus casei*. Multispecies probiotics are defined as containing strains of different probiotic species that belong to one or preferentially more genera, e.g. *Lb. acidophilus*, *Bifidobacterium longum*, *Enterococcus faecium* and *Lactococcus lactis*.

2. Effect of different *Lactobacillus* preparations on growth performance of chickens

Jin et al. (1996) have isolated a total of 42 *Lactobacillus* strains from tissue fragments excised out of the jejunum, ileum and caecum of chickens. The strains were tested in vitro for their ability to adhere to chicken ileal epithelial cells. Twelve strains of the species *Lb. acidophilus*, *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus crispatus* showed moderate to good ability to adhere. A single strain of *Lb. acidophilus* (I 26), which was the most adherent of the 12 strains, and a mixture of the 12 strains were tested in two different experiments. Both probiotic preparations were processed into freeze-dried cultures and subsequently mixed into the diet.

In the first study Jin et al. (1998) investigated the effects of the two probiotic preparations on growth, organ weight, intestinal microflora and intestinal volatile fatty acids (VFA) in broilers. It was hypothesized that the animal-specific *Lactobacillus* strains, by excluding pathogenic bacteria, would enhance chicken performance. The populations of intestinal lactobacilli and coliforms were assessed together with the concentrations of VFA in the ileum and caecum. It was believed that the probiotics would raise VFA levels, thereby decreasing the intestinal pH and creating an unfavourable environment for opportunistic pathogens. In the second study, Jin et al. (2000) looked at the impact of the two probiotic treatments on growth characteristics and the levels of digestive and bacterial enzyme activities in broilers. It was postulated that the ingested probiotics would deliver fermentative enzymes to the gastrointestinal tract and would inhibit growth of putrefactive bacteria. Putrefactive bacteria produce a wide variety of enzymes, such as β-glucu-

Table 1
Body weight, bodyweight gain, feed conversion (feed intake: gain ratio) and mortality of broilers fed a basal diet containing either a monostrain or a multistrain probiotic

Parameter	Study 1			Study 2		
	Control	Probiotic treatment		Control	Probiotic treatment	
		Monostrain	Multistrain		Monostrain	Multistrain
	No supplement	*Lactobacillus acidophilus* I 26	Mixture of 12 *Lactobacillus* strains	No supplement	*Lactobacillus acidophilus* I 26	Mixture of 12 *Lactobacillus* strains
Average initial weight (g)	59.8	59.8	59.5	50.2	50.4	50.2
Average final weight (g)	1349.5	1427.5	1468.8	1632.0[a]	1705.2[a]	1679.5[a]
Average bodyweight gain (g)	1289.7[a]	1367.4[b]	1409.3[b]	1581.8[b]	1654.8[a]	1629.3[a]
Feed conversion (g/g)	2.27[b]	2.17[a]	2.02[a]	2.14[a]	2.03[b]	1.98[b]
Mortality (%)	6.7	8.3	3.3	7.4	7.0	3.9

Source: Compiled from Jin et al. (1998, 2000).
Experimental setup; the three dietary treatments were: (1) basal diet (acted as control); (2) basal diet + 1 g kg^{-1} *Lb. acidophilus* I 26; (3) basal diet + 1 g kg^{-1} mixture of 12 *Lactobacillus* strains.
Bacterial culture preparation; a single strain of *Lb. acidophilus* or a mixture of 12 *Lactobacillus* strains (two *Lb. acidophilus*, three *Lb. fermentum*, one *Lb. crispatus* and six *Lb. brevis* strains) was inoculated into MRS broth and incubated at 37 °C for 24 h. The bacterial cells were harvested by centrifugation, and the bacterial pellets were lyophilized and stored at −20 °C until used. To obtain a concentration of 1 to $2*10^9$ cells per gram, the *Lactobacillus* cultures were diluted with cornstarch and skimmed-milk powder. These dried *Lactobacillus* cultures were stored at 4 °C and mixed into the feed each day. Viability was checked biweekly to ensure that the cultures remained at 1 to $2*10^9$ CFU/g. In total 180 chicks per study were used. Sixty chicks per treatment which were divided over five cages. All means are based on the individual measurements except for the feed to gain ratio which was calculated per cage.
[a,b]Means within study and within rows without common superscript differ significantly ($P<0.05$; GLM SAS).

ronidase and β-glucosidase. Reduction of these noxious bacterial enzymes and an increment of digestive enzyme activity could enhance growth performance and lower mortality of broiler chickens.

The probiotic treatments in both studies significantly increased body weights and decreased feed-to-gain ratios (Table 1). The multistrain preparation, but not the monostrain probiotic, tended to reduce mortality. The first study showed that probiotic treatment induced a significantly lower pH in the caecum associated with an increased concentration of total VFA in ileal and caecal contents. The observed decrease in coliforms in the caecum after 10 and 20 days could be a consequence of the higher VFA level. No effects of probiotics on the relative weights of liver, spleen, bursa, gizzard, duodenum, jeju-ileum and total small intestine were found. In the second study it was found that supplementation with the probiotics significantly increased amylolytic activity in the small intestine. Furthermore, a significant reduction in intestinal β-glucuronidase was seen for both treatments but only the monostrain probiotic significantly reduced the fecal β-glucuronidase activity. The activity of β-glucosidase in the intestine was unaffected, but the activity of this enzyme in faeces was significantly reduced by both treatments.

The results of the two experiments indicate that growth performance of the chickens was improved by both the multi- and monostrain probiotic while the magnitude of the effect was similar. With regard to lowering mortality the multistrain probiotic tended to be more effective than the monostrain probiotic. However, functionality of the multistrain probiotic might be underestimated. During propagation, the 12 *Lactobacillus* strains were incubated together rather than as separate strains. It is likely that some strains were inhibited throughout the fermentation, resulting in an end-product with an unequal distribution of the individual strains. Separate fermentation of the strains followed by mixing of the cultures might enhance the functionality of this kind of multistrain probiotics.

3. Effect of probiotics on fecal bacteria in children treated with the antibiotic ceftriaxone

Zoppi et al. (2001) evaluated the clinical effectiveness of six different commercially available probiotics in preventing or correcting imbalance in the intestinal ecosystem caused by the antibiotic ceftriaxone which was parenterally administered to children to treat upper respiratory tract infections. Use of this antibiotic is known to induce a certain dysbiosis which is characterized by a shift in microbiological numbers representative for the flora of healthy persons (Welling et al., 1991). This shift has a negative impact on colonisation resistance which can result in overgrowth of antibiotic resistant microbes or opportunistic pathogens. Eventually, this may induce clinical symptoms, most commonly (antibiotic associated) diarrhoea (Arvola et al., 1999). As a consequence, the dysbiosis is associated with deviating patterns of fermentative enzyme activities. The products of carbohydrate fermentation (saccharolytic activity) are thought to be beneficial to the host whereas the products of protein fermentation (proteolytic activity) may be potentially toxic. Lactobacilli and bifidobacteria are mainly saccharolytic, resulting in production of short chain fatty acids (SCFAs) which induce a lowering of the intestinal pH and subsequently leads to inhibition of typical proteolytic bacteria (Smith and Macfarlane, 1998). The probiotic bacteria generally have low activities of xenobiotic metabolizing enzymes like β-glucuronidase when compared with *Bacteroides* and *Enterobacteriaceae* (Wollowski et al., 2001).

This study shows that ceftriaxone induces a decrease in *Escherichia coli* and *Lactobacillus* counts and an increase in cocci and *Clostridium* counts. Furthermore, these microbial shifts were associated with a reduction in the activities of fermentative enzymes such as β-galactosidase and β-glucosidase and an increase in the activity of β-glucuronidase, an enzyme involved in the formation of toxic and carcinogenic compounds. From this it was concluded that the parenterally administered ceftriaxone caused a significant dysbiosis.

Six commercial preparations were tested for their ability to reverse the adverse effects caused by the ceftriaxone therapy. Probiotic treatments were administered as freeze-dried preparations in sachets or capsules. Three monostrain probiotics were used: *Saccharomyces boulardii*, *Ec. faecium* SF68 and *Lactobacillus rhamnosus* GG. The following three multistrain/multispecies probiotics were used: a multistrain preparation containing three different *Lactobacillus* strains, namely *Lb. rhamnosus*, *Lb. acidophilus* and

Lactobacillus bifidus (current taxonomy could not be retrieved), a multispecies preparation containing two different species of lactic acid bacteria (*Bifidobacterium bifidum* and *Lb. acidophilus*) and a multispecies preparation, named VSL#3, containing high numbers (as compared to the others) of nine different strains (*Streptococcus thermophilus*, *Ec. faecium*, *Bifidobacterium breve*, *Bifidobacterium infantis*, *B. longum*, *Lb. acidophilus*, *Lactobacillus plantarum*, *Lb. casei* and *Lactobacillus delbrueckii* subspecies *bulgaricus*). A total of 51 children were included in the study, who received either ceftriaxone therapy alone (control) or in combination with one of the probiotics mentioned above. Recorded variables before and after treatment were stool frequency and consistency and the intestinal microflora composition of fecal samples. The fecal samples were also used to measure microbial enzyme activities. Fecal antibiotic resistance was measured to establish whether probiotics affect bacterial resistance as induced by the antibiotic treatment. Bacterial resistance was measured as the occurrence of β-lactamase in the faeces, an enzyme produced by resistant bacteria that inactivates β-lactam antibiotics such as ceftriaxone.

The following observations were made for the monostrain probiotics. *Sacch. boulardii* treatment left the microflora essentially unchanged, except for an increase in fungi (*Sacch. boulardii* is a yeast). The activity of β-glucuronidase was increased by *Sacch. boulardii* treatment, which can be regarded as a potential hazard. Treatment with *Ec. faecium* SF68 did not correct dysbiosis even though it successfully colonized the gastrointestinal tract by replacing the endogenous *Ec. faecium* population. However, the mean anaerobic cocci count was significantly increased and in this respect the dysbiosis caused by ceftriaxone therapy can be considered to be enhanced due to *Ec. faecium* SF68 supplementation. The administration of *Lb. rhamnosus* GG induced favourable alterations in the microflora, but these were less marked than those induced by the multistrain treatments. Both the *Sacch. boulardii* and the *Lb. rhamnosus* GG treatment groups reached the highest percentage of β-lactamase positive samples after treatment, namely 83%. This presence of β-lactamase indicates that there was bacterial resistance towards ceftriaxone and possibly to other β-lactam drugs.

Table 2 gives an overview of the changes induced by the different probiotic treatments as compared to the values before therapy. Only the two multispecies probiotics containing the mixture of lactobacilli and

Table 2
Microbiologic shifts, pH changes and occurrence of antibiotic resistance (measured as β-lactamase activity) in faeces of children with upper respiratory tract infections after treatment with either ceftriaxone alone (control) or combined with different probiotics

Parameter	Control	Probiotic treatment				
		Monostrain		Multistrain	Multispecies	
	No supplement	*Enterococcus faecium*	*Lactobacillus rhamnosus GG*	*Lb. rhamnosus Lactobacillus bifidus Lactobacillus acidophilus*	*Bifidobacterium bifidum Lb. acidophilus*	VSL#3[a]
Aerobic mesophilic count	+1.0 (6.2)	−0.7 (11.5)	+0.2 (10.0)	+1.8 (6.8)	−1.5 (9.6)	+2.2 (9.8)
Escherichia coli	**−2.2[b] (4.4)**	**−4.4[b] (8.4)**	**−6.2[b] (6.8)**	**−3.0[b] (5.6)**	−4.0 (7.7)	**−5.8[b] (8.2)**
Enterobacteria	+0.4 (2.0)	−0.4 (2.7)	−0.2 (2.6)	+0.1 (2.0)	+0.0 (2.0)	−2.4 (4.8)
Anaerobic mesophilic count	+0.4 (7.8)	+0.8 (9.3)	+0.4 (11.4)	+1.3 (9.3)	−1.0 (9.0)	+2.6 (9.4)
Clostridia	+1.4 (4.6)	+0.0 (6.6)	−0.6 (8.4)	+0.5 (6.1)	−1.0 (7.3)	+1.2 (9.2)
Lactobacilli − Bifidobacteria[c]	−0.8 (7.2)	−0.6 (7.6)	−1.2 (8.2)	+1.9 (7.7)	−0.7 (6.7)	+2.0 (8.6)
pH	−0.1 (6.9)	−0.5 (7.2)	−0.6 (6.9)	−0.3 (6.8)	**−0.7[b] (6.8)**	**−0.6[b] (6.9)**
No. of β-lactamase, positive samples	3/5 (0/5)	3/7 (2/7)	6/7 (2/7)	3/7 (1/7)	2/7 (0/7)	2/5 (1/5)

Source: Compiled from Zoppi et al. (2001).

All bacterial counts are expressed as log *n* viable bacteria (CFU) per gram of fresh faeces. Between brackets is the initial mean count of log *n* viable bacteria before therapy. Or in the case of the ratio of β-lactamase positive samples, the ratio of positive samples before therapy.

[a] VSL#3, a preparation containing nine species of lactobacilli, bifidobacteria and streptococci.

[b] Values represented in bold represent statistical significant shifts ($P \leq 0.05$).

[c] Lactobacilli–Bifidobacteria, isolates from anaerobically incubated Rogosa SL agar and MRS agar plates, characterised by morphologic and biochemical analysis.

bifidobacteria significantly counteracted the increase in number of stools per day caused by ceftriaxone therapy (data not shown). The mixture of nine different strains (VSL#3) had the greatest impact on the change in microflora composition as caused by ceftriaxone. No effects of probiotic treatments were found for bacterial enzyme activities. All probiotics studied induced a decrease in stool pH. This decrease can be interpreted as a positive effect because an acidic environment inhibits the growth of pathogenic bacteria and reduces bacterial putrefactive activity. Only two probiotics, both multispecies preparations, were able to induce a statistically significant pH reduction. From this study it can be concluded that probiotics containing multiple species of lactobacilli and bifidobacteria may be more effective in preventing dysbiosis induced by ceftriaxone treatment than other probiotic preparations.

4. Effect of various *Lactobacillus* fermented milks on the severity of a *Salmonella* Typhimurium infection in mice

In the 1980s Perdigon and her colleagues have published numerous studies on the effect of *Lactobacillus* fermented milk on the immune system in mice. In one study Perdigon et al. (1990) tested the protective effect of milk fermented with either *Lb. acidophilus*, *Lb. casei* or a combination of both strains in mice challenged with *Salmonella* Typhimurium. Mice were fed for 8 days one of the fermented products followed by an oral challenge with *S.* Typhimurium. The fermented milks were administered as a 20% suspension in the drinking water, resulting in a total of $2.4*10^9$ viable organisms administered per day. The control group received a 10% solution of skim milk powder mixed with the drinking water in a 1:4 ratio. Survival of the mice was followed for 21 days. The number of viable salmonellae in liver and spleen was determined at different time intervals and so were serum and intestinal fluid antibodies concentrations against *S.* Typhimurium.

The results are given in Table 3. The monostrain fermented milks failed to enhance resistance towards *S.* Typhimurium, although the initial survival rates were higher than those of the controls. The monostrain preparations with *Lb. casei* induced a significant reduction in salmonellae counts in liver and spleen on day 10 after challenge and produced an almost two-fold higher serum antibody titre than seen in the controls. *Lb. acidophilus* treated mice showed the lowest antibody titres in both serum and intestinal fluid.

It is clear that only pre-treatment with multistrain fermented milk was effective in preventing colonization of *S.* Typhimurium in liver and spleen. On day 7

Table 3
Survival, number of viable salmonellae in liver and spleen and the levels of specific antibodies in serum and intestinal fluid of mice challenged with *Salmonella* Typhimurium and fed either skim milk powder or milk fermented with either *Lactobacillus casei*, *Lactobacillus acidophilus* or a combination of both strains

Day after challenge	Control	Probiotic treatment		
		Monostrain		Multistrain
	Skim milk powder	*Lactobacillus casei*	*Lactobacillus acidophilus*	*Lb. casei* + *Lb. acidophilus*
Survival rates of mice fed different fermented milks[a]				
0	100%	100%	100%	100%
7	20%	97%	80%	100%
15	20%	77%	30%	100%
21	20%	20%	20%	100%
Number of viable salmonellae in livers/spleens[b]				
1	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
2	0.0/0.0	3.9/4.1	4.7/4.2	0.0/0.0
3	0.0/0.0	4.4/4.1	0.0/0.0	3.3/3.3
4	3.0/2.3	4.1/3.9	0.0/0.0	3.7/2.4
7	5.2/4.6	2.4/2.1	3.2/2.7	0.0/0.0
10	3.3/3.9	1.5/1.2	2.9/2.4	0.0/0.0
Levels of anti-Salmonella Typhimurium antibodies in serum/intestinal fluid[c]				
4	278/226	370/545	139/82	833/464
7	617/150	1049/1064	324/55	1991/545
10	355/55	741/286	253/82	2531/232
15	139/55	309/218	93/82	627/164

Source: Compiled from Perdigon et al. (1990).
Significance and the number of animals per group or measurement were not exclusively mentioned in the reviewed article; these are therefore not included in this table.

[a] This group received a more lethal dose of 40_{LD50} *S.* Typhimurium.

[b] This group received a dose of 20_{LD50} *S.* Typhimurium; number of salmonellae in liver or spleens is expressed as log viable bacteria per organ.

[c] This group received a dose of 20_{LD50} *S.* Typhimurium; levels of antibodies against salmonellae are expressed as the highest serum or intestinal fluid dilution giving a positive agglutination reaction.

after challenge viable salmonellae had disappeared from the liver and spleen. This was associated with a 100% survival of the mice in the *Lb. casei*+*Lb. acidophilus* group. Serum antibodies against *S.* Typhimurium in mice fed multistrain fermented milk were higher than in the other groups. The successful multistrain treatment may be the result of an optimal combination of strain-specific properties such as activation of the specific immune response by *Lb. casei* (Table 3) and the induction of non-specific immune responses by *Lb. acidophilus* shown in another study published by the same group (Perdigon et al., 1987). In any event, the data in Table 3 show convincingly that the combination of *Lb. casei* and *Lb. acidophilus* provided protection against *S.* Typhimurium whereas the bacterium strains alone did not.

5. Effect of *Lb. casei* strains alone or in combination on survival in mice challenged with *Salmonella* Typhimurium

Paubert-Braquet et al. (1995) used mice orally infected with *Salmonella* Typhimurium to test the protective effect of milks fermented with different strains of the *Lb. casei* species, yogurt ferments or a combination of both kinds of ferments. The bacterial contents of the test preparations are presented in Table 4 (see footnote). Mice were supplemented for a 7-day period with one of the fermented milks, standard milk or received no supplement. Then the mice were orally infected with *S.* Typhimurium and the survival was monitored daily for 14 days. The phagocytosis index was determined by injecting colloidal carbon into the tail vein and measuring its clearance from the blood. Furthermore, serum IgA levels and β-glucuronidase (in this regard a bactericidal enzyme produced by macrophages) activity in the supernatant of peritoneal macrophages were measured.

Table 4 shows that milks fermented with different species of the *Lactobacillus* genus induced different protection levels against *S.* Typhimurium. Irrespective of the type of fermented milk administered, survival in all treatment groups was significantly higher than in the control group supplemented with standard milk. Most protection was provided by the mixture (Yogurt ferment and LAB-1), causing 87.5% survival after 14 days. The protective effect induced by the multispecies ferment did not reflect the immunomodulating variables. Only the monostrain fermented milk with LAB-1 and the Shirota strain induced a significant increase in the phagocytosis index when compared to the mice fed standard milk. The group supplemented with the yogurt ferment produced a significantly lower index than that seen in the group given standard milk. Likewise, only the LAB-1 and Shirota-strain

Table 4
The effect of five different fermented milks versus either no supplement or non-fermented milk on survival of mice infected with *Salmonella* Typhimurium

Day after challenge	Control		Probiotic treatment				
			Monostrain			Multispecies	
	No supplement	Non-fermented milk	*Lactobacillus casei* (LAB-1)	*Lb. casei* (LAB-2)	*Lb. casei* strain Shirota	Yogurt ferment	*Lb. casei* (LAB-1)+yogurt ferment
2	100%	100%	100%	100%	100%	100%	100%
6	62.5%	62.5%	87.5%	62.5%	62.5%	75%	87.5%
10	25%	37.5%	75%	50%	50%	50%	87.5%
14	0%	12.5%	75%[a,b]	50%[a]	50%[a]	50%[a]	87.5%[a,b]

Source: Compiled from Paubert-Braquet et al. (1995).

Experimental setup; each treatment group consisted of eight animals. Animals were supplemented for 7 days with non-fermented milk or fermented milks at a rate of 30% of their normal daily diet. The various probiotic treatments contained the following amounts of bacteria (CFU/ml): LAB-1, 2.5×10^8 of *Lb. casei*; LAB-2, 4.9×10^8 of *Lb. casei*; *Lb. casei* strain Shirota, 1.0×10^8 of *Lb. casei*; yogurt ferment, 8.3×10^8 of *Streptococcus thermophilus*+1.1×10^8 of *Lactobacillus bulgaricus*; LAB-1+yogurt ferment, 1.1×10^8 of *St. thermophilus*+8.2×10^8 of *Lb. bulgaricus*+0.8×10^8 of *Lb. casei*.

[a] On day 14 all probiotic treatments showed significantly higher survival rates than those observed in the standard milk group.

[b] Survival rates in the LAB-1 group and the yogurt ferment+LAB-1 group were significantly higher than those observed in the LAB-2, yogurt ferment and *Lb. casei* strain Shirota treated group.

fermented milk induced significantly higher levels of serum IgA and of β-glucuronidase in the supernatant of peritoneal macrophages when compared to the standard-milk group. It can be concluded that the effects of the probiotics on immunomodulating variables were not associated with those on survival. Nevertheless, it is clear that the combination of *Lb. casei* and yogurt ferment offered most protection against *S.* Typhimurium challenge.

6. Efficacy of different probiotic bacteria in reducing *E. coli* O157:H7 shedding by sheep

E. coli O157:H7 is an enterohemorrhagic type of *E. coli* commonly implicated in human food-borne illness. This serotype is particularly dangerous because of its low infectious dose, and its unusual acid tolerance. *E. coli* O157:H7 is frequently harboured in apparently healthy ruminants. It has been suggested that the fasting of ruminants just before slaughter can induce an increase in ruminal fluid pH because of a lack of easily fermentable sugars for microbial acid production, resulting in optimal conditions for unrestricted growth of *E. coli* O157:H7. This causes a higher risk of contaminating the meat during slaughter. Previous studies (Zhao et al., 1998) have indicated that *E. coli* O157:H7 shedding can be reduced by inoculating ruminants with certain probiotic bacteria prior to infection.

Lema et al. (2001) have studied the efficacy of *Lb. acidophilus*, *Ec. faecium, Lb. casei*, *Lb. fermentum* and *Lb. plantarum* as to reduce *E. coli* O157:H7 shedding by sheep already infected earlier with the pathogen. Two monostrain preparations containing either *Lb. acidophilus* or *Ec. faecium* were tested and also two multispecies preparations containing a mixture of *Lb. acidophilus* and *Ec. faecium* or a mixture of *Lb. acidophilus*, *Ec. faecium*, *Lb. casei, Lb. fermentum* and *Lb. plantarum*. The microbial supplements were composed of freeze-dried fermentation products of the bacteria and contained $2*10^9$ CFU of microorganisms per gram of product. Thirty Suffolk ram lambs were inoculated with a 1-ml suspension of 10^{10} CFU of *E. coli* O157:H7, starting on the same day as probiotic treatment and the probiotic treatment continued thereafter for 7 weeks. The control group received a basal diet without microbial supplements. The four experimental groups received the same basal diet supplemented daily by mixing one of the four microbial treatments with the diet at a rate of 0.3 g/kg diet ($6.0*10^6$ CFU/kg diet). The feed was offered to the lambs for ad libitum consumption. Fresh faeces were retrieved from the rectum every week directly followed by selective enumeration of *E. coli* O157:H7. Fecal consistency was scored at the time of sampling in order to see whether the animals had diarrhoea. Animal performance variables such as feed consumption (FC), gain-to-feed ratio (G/F) and average daily weight gain (ADG) were monitored for the entire experimental period.

All lambs remained clinically healthy throughout the experimental period without evidence of diarrhoea. Lambs that were administered the mixture of *Lb. acidophilus*, *Ec. faecium*, *Lb. casei*, *Lb. fermentum* and *Lb. plantarum* shed significantly lower numbers of *E. coli* O157:H7 in the faeces than did the other groups and this held for the entire experimental period (Table 5). As to monostrain preparations, no effect of *Lb. acidophilus* supplementation was observed when compared to the control. In contrast, *Ec. faecium* supplementation produced a significantly lower mean count of *E. coli*. The combination of *Lb. acidophilus* and *Ec. faecium* did not reduce *E. coli* shedding. In conclusion, the multispecies preparation containing five strains was more effective than the two-strain or monostrain preparations. From Table 5 it can be suggested that beyond the 7-week duration of the experimental period only the multispecies preparation containing five strains may provide successful clearance. Both multispecies preparations had a significantly positive effect on average daily growth and the feed-to-gain ratio when compared to the control and the monostrain groups.

It is important to note that the probiotic effect of *Ec. faecium* as to *E. coli* shedding was counteracted by the addition of *Lb. acidophilus*. On the other hand, the same combination did enhance gain-to-feed ratio and average daily gain when compared with the administration of either *Ec. faecium* alone or *Lb. acidophilus* alone. It could be suggested that not only strain-specific properties exist but also combination-specific properties. In conclusion, supplementing lambs with *Ec. faecium* reduced fecal *E. coli* O157:H7 shedding, but more effective reduction was obtained by treatment with the multispecies

Table 5
The effect of four different microbial supplements versus no supplement on fecal *E. coli* O157:H7 shedding, feed consumption, average daily weight gain and feed conversion in sheep infected earlier with *E. coli* O157:H7

	Control	Probiotic treatment			
		Monostrain		Multispecies	
	No supplement	*Lactobacillus acidophilus*	*Enterococcus faecium*	*Lb. acidophilus*, *Ec. faecium*	*Lb. acidophilus*, *Ec. faecium*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus plantarum*
Faecal E. coli O157:H7 shedding after start of probiotic treatment (log_{10} CFU/g of faeces)					
Week 1	5.8[a]	4.2[a]	2.8[b]	2.1[b]	2.1[b]
Week 3	6.2[a]	6.3[a]	5.5[a]	5.1[a]	3.2[b]
Week 5	6.8[a]	6.8[a]	2.8[c]	5.0[b]	2.6[c]
Week 7	4.9[a]	5.0[a]	3.1[b]	4.8[a]	1.0[c]
Feed consumption (FC), average daily weight gain (ADG) and feed conversion (FC/ADG ratio)					
FC (g/day)	500.0[a]	500.0[a]	470.0[a]	461.0[a]	500.0[a]
ADG (g)	163.0[b]	186.4[b]	186.2[b]	213.6[a]	219.1[a]
Feed conversion (g/g)	3.07[b]	2.68[b]	2.52[b]	2.16[a]	2.28[a]

Source: Compiled from Lema et al. (2001).
Experimental setup; the five dietary treatments were: (1) basal diet (acted as control); (2) basal diet + 0.3 g kg^{-1} *Lb. acidophilus*; (3) basal diet + 0.3 g kg^{-1} *Ec. faecium*; (4) basal diet + 0.3 g kg^{-1} *Lb. acidophilus* + *Ec. faecium*; (5) basal diet + 0.3 g kg^{-1} *Lb. acidophilus* + *Ec. faecium* + *Lb. faecium* + *Lb. casei* + *Lb. fermentum* + *Lb. plantarum*.
Microbial feed supplements were purchased from Chr. Hansen BioSystems. The microbial supplements were composed of freeze-dried fermentation products of the bacteria, dried whey, sodium sulfate and sodium silico aluminate and contained $2.0*10^9$ CFU of microorganisms per gram of product. Viability was checked to ensure that the cultures contained $2*10^9$ CFU/g.
Lambs were blocked by body weight (six blocks of five lambs each) and lambs within the block were randomly assigned to the five different dietary treatments.
[a,b,c]Different letters a, b and c within rows indicate significantly different values ($P < 0.05$).

preparation containing *Lb. acidophilus*, *Ec. faecium*, *Lb. casei*, *Lb. fermentum* and *Lb. plantarum*.

7. The effect on growth of mono- versus multistrain/multispecies probiotics in rats challenged with *Salmonella* Enteritidis

We have conducted an experiment in rats challenged with *Salmonella* Enteritidis to compare the protection induced by a monostrain probiotic versus that induced by multistrain and/or multispecies probiotics (Van Es and Timmerman, 2002). In the experiment male Wistar (U-WU) rats were challenged with a single oral dose of $1.0*10^9$ *S.* Enteritidis. Before challenge the rats were trained to ingest their restricted amount of daily feed within 1 h. The diets were administered as freshly prepared porridges mixed with different probiotic cultivars (Table 6). The control animals were fed a diet mixed with fermentation broth containing heat-killed *Lb. casei*. All the other animals received with their diet a total of $1*10^9$ CFU of different probiotic organisms per day. Multistrain probiotics were individually grown and then mixed. All probiotic preparations were microbiologically enumerated to check their viable numbers. The animals were challenged with an anticipated sublethal dose of *S.* Enteritidis, none of the rats died and no signs of disease were seen. *Salmonella* could be cultured from the faeces collected (data not shown) so that it was concluded that the virulence of the strain was not high enough to induce systemic complications. However, we did see treatment differences in weight gain throughout the post-challenge period. A distinction can be made between weight loss as a consequence of the infection with *S.* Enteritidis, subsequent recovery and post-challenge weight gain.

This kind of *Salmonella* infection-associated weight changes has also been described by Gill et al. (2001). They performed a similar experiment in which the protective effect of *Lb. rhamnosus* strain HN001 against translocation of *Salmonella* Typhimu-

Table 6
The effect of different dietary probiotic treatments on weight change in rats after challenge with *Salmonella* Enteritidis

Parameter	Control	Probiotic treatment				
		Monostrain	Multistrain		Multispecies	
	Lactobacillus casei (dead)	*Lb. casei* (alive)	*Lb. casei* + *Lactobacillus acidophilus*	*Lb. casei* + *Lb. acidophilus* + *Lactobacillus salivarius*	*Lb. casei* + *Lb. acidophilus* + *Lb. salivarius* + *Lactococcus lactis*	*Lb. casei* + *Lb. acidophilus* + *Lb. salivarius* + *Lc. lactis* + *Lactobacillus plantarum*
Immediate post-challenge weight change (severity of infection), day 0–day 3 (g)[†]	−4.63	−2.50	−4.5	−1.25	0.38	−0.75
Growth recovery, day 3–day 9 (g)[†]	5.13	7.13	12.13	10.25	12.25	5.88
Overall post-challenge growth, day 0–day 9 (g)[†]	0.50[a]	4.63	7.63	9.00	12.63[b]	5.13

Source: Compiled from Van Es and Timmerman (2002).
Each treatment group consisted of eight animals. The challenge was conducted on day 0.
[a,b]Different letters a and b within rows indicate significantly different values ($P < 0.05$).
[†] Numbers represent the average weight change per treatment.

rium in mice was tested. After a single oral dose of 10^7 *S.* Typhimurium the general health score (GHS; a 1–5 score index for the clinical appearance) was recorded daily as well as the food and water intake, and weight change. Changes in the GSH became evident on day 5 post-challenge and the scores had fallen noticeably on days 6 and 7. Mortality was first seen on day 6 in the controls, and among the probiotic-treated animals only on day 10. Weight change during the first 7 days post-challenge was significantly different between the two groups, the probiotic-treated animals gaining weight and the control animals losing weight. Also in our experiment with rats we found that 3 days after challenge animals gained weight again. During the first 3 days post-challenge mean weight reduction was lowest in the animals receiving more than two different probiotic strains (Table 6). Post-challenge weight gain was highest for the group treated with the mixture of *Lb. casei*, *Lb. acidophilus*, *Lb. salivarius* and *Lc. lactis*. Interestingly, growth recovery and overall growth were lower if *Lb. plantarum* was added to these four species. This observation led us to conclude in agreement with Lema et al. (2001) that certain combinations of probiotic strains are not beneficial and lead to diminished efficacy.

Probiotics are appreciated for their antimicrobial activity, but this property may also be a potential weakness for probiotic mixtures. Secreted antimicrobial compounds such as lactic acid, hydrogen peroxide and bacteriocins not only inhibit potential pathogens but also closely related species (Kailasapathy and Chin, 2000). Therefore we conducted a series of in vitro experiments to determine whether inhibitory activity exists between strains and whether this correlates with in vivo efficacy. In short, individual strains were co-cultured with all other strains according to the cross-streak method as applied in the CAMP-test (Smibert and Krieg, 1994). *Lb. acidophilus*, *Lb. salivarius* and *Lc. lactis* showed no inhibitory activity towards the other individual strains. *Lb. casei* inhibited growth of *Lb. acidophilus* and *Lb. salivarius* but not of *Lc. lactis*. It was this combination that showed the highest efficacy in the in vivo study. *Lb. plantarum* inhibited all strains except *Lc. lactis*. This strong in vitro inhibition tended to mimic the in vivo situation, in which addition of *Lb. plantarum* clearly inhibited in vivo efficacy of the four-strain mixture (Table 6).

8. Possible mechanisms involved in multispecies probiotics

Knowing that health effects of probiotics are genera, species and strain specific (Sanders and Huis

in't Veld, 1999) it could be suggested that multistrain and/or multispecies probiotics may be more effective than monostrain probiotics. In this review it is investigated whether probiotics consisting of more than one strain of the same species or genus (named multistrain or multispecies probiotics) are superior to monostrain probiotics. The studies described indeed provide evidence for multistrain probiotics being more effective than monostrain probiotics. The use of multispecies preparations, containing multiple strains of more than one genus, could even be more effective than that of multistrain probiotics. The multispecies probiotic VSL#3 has been shown to be superior to the 'traditional' therapies with antibiotics or 'conventional' monostrain probiotics in the treatment of pouchitis and ulcerative colitis (Gionchetti et al., 2002; Shibolet et al., 2002; Ulisse et al., 2001). The superiority of multistrain or multispecies probiotics, when compared with monostrain probiotics, is evident for different preparation techniques such as fermented products (Paubert-Braquet et al., 1995; Perdigon et al., 1990) and freeze-dried cultures (Jin et al., 1998, 2000; Lema et al., 2001; Zoppi et al., 2001).

The studies described were not specifically designed to compare the efficacy of multistrain or multispecies probiotics with that of monostrain probiotics. Thus, the mechanisms underlying the enhanced effects of these probiotic mixtures remain obscure. Table 7 summarizes factors that may positively influence the efficacy of multispecies probiotics when compared to monostrain probiotics. One well-known health effect of probiotics is that they can fortify colonization resistance (CR) in the intestinal ecosystem against potential pathogens. However, the probiotic itself first has to overcome CR exerted by the resident microflora once it is ingested. Furthermore, host properties, such as an acidic environment in the stomach, bile acids and pancreatic enzymes in the duodenum, determine to what extent the probiotic will survive. With probiotic preparations containing different strains there will be an increased chance of at least partial survival since there may be strains that are less affected. Survival rates of 20–40% have been estimated for selected strains (Bezkorovainy, 2001). Multistrain probiotics may be able to create a probiotic niche which enhances colonization of 'damaged' strains. Strains with an optimal pH range of 6–7 (pH upper intestinal tract) may display rapid growth, causing a local decline in pH and thereby creating the optimal pH range of more acidophilic bacteria in the probiotic. Certain probiotic species are dependent on other strains for their carbohydrate supply. For example, *Lactobacillus* strains produce mainly lactate which is catabolized by propionibacteria into propionic acid (Frohlich-Wyder et al., 2002). In vitro data indicate two different mechanisms that may be beneficial for multispecies probiotics in creating their

Table 7
Overview of differences between monostrain probiotics and multispecies probiotics as to their successful colonisation and subsequent health promoting effects

Monostrain probiotic	Multispecies probiotics
Successful colonisation	
Survival depends on the properties of one specific strain • This strain has to overcome on its own all barriers exploited by the host and its endogenous microflora	Different strains with different characteristics have an enhanced chance of colonization • Greater divergency of strong points; enhanced chance of survival of at least one or several strains • Creation of a probiotic niche; improving chances of successful colonisation of the other strains, through, e.g. ○ Reduction of antagonistic activity of the endogenous microflora against other sensitive probiotic strains ○ Induction of an optimal pH range ○ Creation of an anaerobic niche ○ Enhanced adhesion
Health effects exerted by the probiotic preparation	
Probiotic effect is limited to the strain specific properties	Probiotic effect enhanced due to combination of strain specific properties • Additive effect of specific strain properties such as colonization of different niches • Synergistic effects of different strains with specific properties; the total probiotic effect may be more than the sum of the separate health promoting properties Positive interrelationships between strains which enhance their biological activity • Symbiosis between different strains, e.g. due to exchange of different metabolites

own probiotic niche. First, certain strains like *S. thermophilus* are oxygen scavengers and create anaerobic conditions that could enhance the growth and survival of strict anaerobes like bifidobacteria (Shankar and Davies, 1976). Secondly, the ability to adhere to mucosal surfaces is related to various probiotic health effects, and it is regarded as a prerequisite for stimulation of the immune system and for antagonistic activity against enteropathogens (Ouwehand et al., 2000). The ability of different strains and their mixtures to adhere to human intestinal mucus was studied in vitro. Surprisingly, it appeared that certain combinations showed synergistic effects. The presence of *Lb. rhamnosus* GG or *Lb. delbrueckii* subsp. *bulgaricus* more than doubled the adhesion of *Bifidobacterium animalis* BB-12, while the adhesion of *Propionibacterium freudenreuchii* P6 was more than tripled by the presence of *Lb. rhamnosus* GG and almost doubled by the presence of *B. animalis* BB-12 (Ouwehand et al., 2000, 2002b). The feature of stimulation of adhesion of one strain by another greatly enhances successful colonization of multistrain probiotics. This also holds for promising probiotic species such as representatives of the *Propionibacterium* genus which by themselves would be considered as non-probiotic because of their low adhesiveness.

The advantage of multistrain and multispecies probiotics is that a number of favorable characteristics of individual strains are combined in a single preparation (Campieri and Gionchetti, 1999). This may be particularly relevant for users with a variety of gastrointestinal complaints. However, it is also obvious from other considerations and experimental results. One consideration relates to the specific niche of probiotic bacteria: whereas *Lactobacillus* is the most abundant member of the LAB-genera in the proximal small intestine, *Bifidobacterium* has a strong preference for the large intestine. Experimental evidence for enhanced efficacy of multistrain probiotics against gastrointestinal pathogens comes from Drago et al. (1997) and from Apella et al. (1992). Drago et al. (1997) have tested three promising newly isolated human *Lactobacillus* strains as to their individual and combined activity against selected enteropathogens (*E. coli*, *Salmonella* Enteritidis and *Vibrio cholerae*). Measures were taken to rule out inhibition by pH variation or nutrient consumption. Only the mixture of the three *Lactobacillus* strains was able to almost completely inhibit the growth of *E. coli* and *S.* Enteritidis, whereas no significant inhibition of *V. cholerae* growth was observed. Apella et al. (1992) found similar results in regard to the inhibitory effect of lactobacilli on growth of *Shigella sonnei*. The increased efficacy of multistrain probiotics against pathogens may be caused by the greater variety of antimicrobial capacities associated with mixed preparations, such as production of weak organic acids, bacteriocins, hydrogen peroxide, coaggregation molecules (blocks the spread of the pathogen) and/or biosurfactants (inhibit adhesion), and the stimulation of sIgA production and mucus secretion by the host (see also Table 7).

A part of the additive and synergistic health-promoting effects of individual strains in multistrain probiotics may be explained from possible interrelationships between strains in these mixtures. Symbiosis may enhance certain probiotic characteristics like growth or metabolic activity of strains (see Table 7). Growth of the probiotic organism is necessary to maintain sustainable numbers at a certain site in the gastrointestinal tract. This growth can be stimulated by the presence of other strains as is known for certain starter cultures in the manufacture of fermented dairy products (Gomes et al., 1998; Kailasapathy and Chin, 2000; Warminsko-Radyko et al., 2002). For probiotic bacteria such as *Lb. acidophilus* and *Bifidobacterium* spp., it is known that they grow slowly in milk because they lack proteolytic activity. Addition of typical yoghurt bacteria particularly *Lb. delbrueckii* subsp. *bulgaricus* will enhance the growth of the probiotic strains (Shihata and Shah, 2000). The positive interaction between strains was referred to by Driessen et al. (1982) as protocooperation and is explained by the exchange of certain growth factors, such as amino acids, free peptides, formate and CO_2. Gomes et al. (1998) reported a progressive increment of *B. animalis* growth through the presence of *Lb. acidophilus* which hydrolyzes milk caseins using extracellular proteinases and yielding amino acids and peptides that stimulate the growth of *B. animalis*. On the other hand, growth of *Lb. acidophilus* can also be enhanced by the presence of *B. animalis*, possibly due to the production of acetate (Kailasapathy and Chin, 2000).

Another probiotic bacterium used in the manufacture of Swiss-type cheeses, *P. freudenreuchii* 7025, produces 2-amino-3carboxy-1,4-naphthoquinone that enhances the growth of bifidobacteria (Mori et al.,

1997). Whereas growth of propionibacteria can be stimulated through peptides produced from casein by *Lactobacillus helveticus* (Piveteau et al., 2000). Lactobacilli are also able to produce bifidogenic growth factors in the form of extracellular polysaccharides (EPS). EPS may protect the microorganism against anti-microbial factors because it surrounds the bacterial cell as a capsule or is secreted into the extracellular environment as slime. Surprisingly, EPS produced by *Lc. lactis* subsp. *cremoris* cannot be used as an energy source by the bacterium itself (Looijesteijn et al., 2001). However, EPS produced by *Lactobacillus sanfranciscencis* serve as a prebiotic or bifidogenic growth factor for bifidobacteria (Bello et al., 2001). Together with growth, metabolic activity is also influenced by symbiotic relationships. Sodini et al. (2000) have identified interacting mixed cultures of lactic acid bacteria through the use of a mathematical model. The acidifying activity of mixed cultures was predicted on the basis of acidification tests conducted with the pure cultures. In the case of underestimation of acidifying activity by the designed model, a positive interaction between the strains was assumed. Different combinations of *S. thermophilus* and *Lb. delbrueckii* strains were tested. Only two positive interactive mixtures were found, suggesting that symbiotic relationships are generally not on the species level, but rather on the strain level. It can be generally concluded that different strains of the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Bifidobacterium* and *Propionibacterium* show symbiotic relationships towards each other which enhances growth and metabolic activity. Furthermore, it can be expected that this enhanced probiotic activity causes an increased nutrient consumption, a well-known probiotic mechanism in the control of intestinal pathogens. The use of positively interacting strains of these genera in multistrain or multispecies probiotics should be encouraged.

9. Conclusive remarks

With this review we tried to show the relevance of developing multispecies probiotics which may have improved functionality as compared to single strain probiotics. It is clearly shown that multispecies preparations have advantages when compared to monostrain probiotics or, to a lesser extent, multistrain probiotics. Well-designed multispecies probiotics can benefit from a certain amount of synergism when different probiotic effects of different probiotic species are combined. The activity can also be stimulated through symbiosis among strains in the preparation. We recommend further research on multispecies preparations in which combinations of strain-specific properties are chosen to be additive or synergistic. In vitro research should aim at finding combinations which show synergistic and symbiotic activities towards each other to maximize the chance of providing clinically more effective probiotic preparations. Special attention should also be paid to avoid combinations of probiotic strains showing mutual inhibitory properties, e.g. through the production of H_2O_2, bacteriocins or bacteriocin-like substances (Kailasapathy and Chin, 2000).

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