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Review article: bifidobacteria as probiotic agents – physiological effects and clinical benefits

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SUMMARY

Bifidobacteria, naturally present in the dominant colonic microbiota, represent up to 25% of the cultivable faecal bacteria in adults and 80% in infants. As probiotic agents, bifidobacteria have been studied for their efficacy in the prevention and treatment of a broad spectrum of animal and/or human gastrointestinal disorders, such as colonic transit disorders, intestinal infections, and colonic adenomas and cancer. The aim of this review is to focus on the gastrointestinal effects of bifidobacteria as probiotic agents in animal models and man. The traditional use of bifidobacteria in fermented dairy products and the GRAS ('Generally Recognised As Safe') status of certain strains attest to their safety. Some strains, especially *Bifidobacterium animalis* strain DN-173 010 which has long been used in fermented dairy products, show high gastrointestinal survival capacity and exhibit probiotic properties in the colon. Bifidobacteria are able to prevent or alleviate infectious diarrhoea through their effects on the immune system and resistance to colonization by pathogens. There is some experimental evidence that certain bifidobacteria may actually protect the host from carcinogenic activity of intestinal flora. Bifidobacteria may exert protective intestinal actions through various mechanisms, and represent promising advances in the fields of prophylaxis and therapy.

INTRODUCTION

The human large intestine is a densely populated microbial ecosystem. Several hundred species of bacteria are usually present and the total weight of microbiota living within the colonic lumen is estimated to be several hundred grams.[1] There are up to 10^{13}–10^{14} total bacteria in the human intestinal tract, i.e. 10- to 20-fold more than the total number of tissue cells in the entire body.[2] Most of the bacteria are obligate anaerobes, including clostridia, eubacteria, bacteroides groups and the genus *bifidobacterium*, such as *Bifidobacterium bifidum* and *Bifidobacterium infantis*. *Bifidobacterium* is a member of the dominant microbiota (i.e. $>10^8$–10^9 colony forming unit (CFU)/g using culture methods, >1% of the total bacteria count using molecular biology methods), both in human faeces (3.2% ± 0.55 of total bacterial rRNA) and in the content of the caecal lumen (5.2 ± 0.37%) as shown by culture and molecular hybridization using rRNA-targeted probes or quantitative PCR.[3–6] Table 1 shows the distribution of bifidobacteria species in the intestinal flora of human adults as evaluated by quantitative PCR.

It is a long-standing belief, which probably originated with Metchnikoff at the turn of the 20th century, that some gut bacteria are beneficial to health, whilst others may be harmful. Obviously, some gut bacteria are harmful in that they produce toxins causing diarrhoea,

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Table 1. Distribution of *Bifidobacterium* species in the intestinal flora of human adults as evaluated by quantitative PCR. Adapted from Matsuki *et al.*.[5] Log_{10} bifidobacteria/g of faeces measured by reaction with genus- or species-specific primer*

Species	Genus *Bifidobacterium*	*B. adolescentis*	*B. angulatum*	*B. bifidum*	*B. breve*	*B. catenulatum*	*B. longum*	*B. infantis*
No. positive (%)	46 (100)	38 (83)	5 (11)	13 (28)	8 (17)	41 (89)	44 (96)	2 (4.3)
Mean ± s.d.	9.4 ± 0.7	9.1 ± 0.9	6.6 ± 0.2	8.3 ± 0.8	7.3 ± 0.7	8.9 ± 0.8	8.1 ± 0.7	6.9 ± 0.7
Range in positive subjects	[6.9; 10.6]	[7.4; 10.6]	[6.3; 6.9]	[6.8; 9.4]	[6.4; 8.4]	[6.3; 10.2]	[6.4; 9.4]	[6.4; 7.3]

* Minimum detection threshold of the method used: 6 Log_{10} CFU/mL.

mucosal invasion and activation of carcinogens is self-evident. Such bacteria are thought to include some *Clostridium* spp., sulphate-reducing and amino acid-fermenting species. The main potentially health-enhancing bacteria are the bifidobacteria and lactobacilli, both of which belong to the lactic acid bacteria (LAB) group.[7] These two genera do not include any significant pathogenic species and their dominance in the faeces of breast-fed babies is thought to impart protection against infection.[8, 9] The health interest of the *Bifidobacterium* genus is reflected in the commonly-accepted definition of prebiotics: food ingredients that selectively stimulate the growth and activity of bacteria in the gut, usually bifidobacteria (bifidogenic effect) and lactobacilli thus procuring health benefits.[10, 11]

The aim of this review is to focus on the physiological effects of health-promoting bifidobacteria. When considering the study of one specific strain, most of relevant scientific data on Bifidobacteria are focused on *Bifidobacterium animalis* DN-173 010. For this reason, this species has been used as a reference in this review.

BIFIDOBACTERIA: SAFETY IN USE

The safe use of bifidobacteria is supported by the long historical consumption of fermented milks and the growing knowledge about bifidobacteria taxonomy and physiology.[12, 13] Lactic acid-producing bacteria in foods are considered as commensal microorganisms with little or no pathogenic potential.[14] Indeed, a recent review of the safety of lactobacilli and bifidobacteria used as probiotics concluded that they posed no health risks for consumers.[15]

Regarding taxonomy, modern molecular techniques, including polymerase chain reaction-based and other genotyping methods, have become increasingly important for species identification and for the differentiation of bifidobacteria strains.[16]

'16S rRNA sequence analysis (usually used to produce phylogenic trees) is not suitable to distinguish different species of *Bifidobacterium*.[17] So, the gene sequence of heat-shock protein of 60 kDa (HSP 60) is preferentially used; furthermore, it is found as a single copy in almost all bacterial species. The phylogenic tree is realized comparing a DNA fragment of 0.6 kb of the HSP 60 of each studied *Bifidobacterium* species. The more the sequences are close (in term of percentage of similarities), the more the species are close on the tree'. As an example, Figure 1 presents details about the phylogeny of *B. animalis*.

It should be noted that this recognition of the safety of such strains will be formalized in a European regulatory framework that is in the process of defining the criteria to be evaluated when assessing the safety of microorganisms used in the food and feed industry.[18]

THE PROBIOTIC CONCEPT

Probiotics are defined as 'live micro-organisms which confer a health benefit on the host when administered in adequate amounts'.[19] They have been widely tested, in animal and human studies, for their beneficial actions in the prevention or treatment of a broad spectrum of gastrointestinal disorders, from impairment of colonic transit to colonic carcinogenesis. Other functional foods include prebiotics and synbiotics. As already mentioned, prebiotics are defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon.[20] Synbiotics are products in which both a probiotic and a prebiotic are combined.

Some bifidobacteria strains which are used in fermented milks show high survival in the gastrointestinal tract and exhibit probiotic properties in the colon, thus fulfilling therefore criteria for probiotics.[21–23]

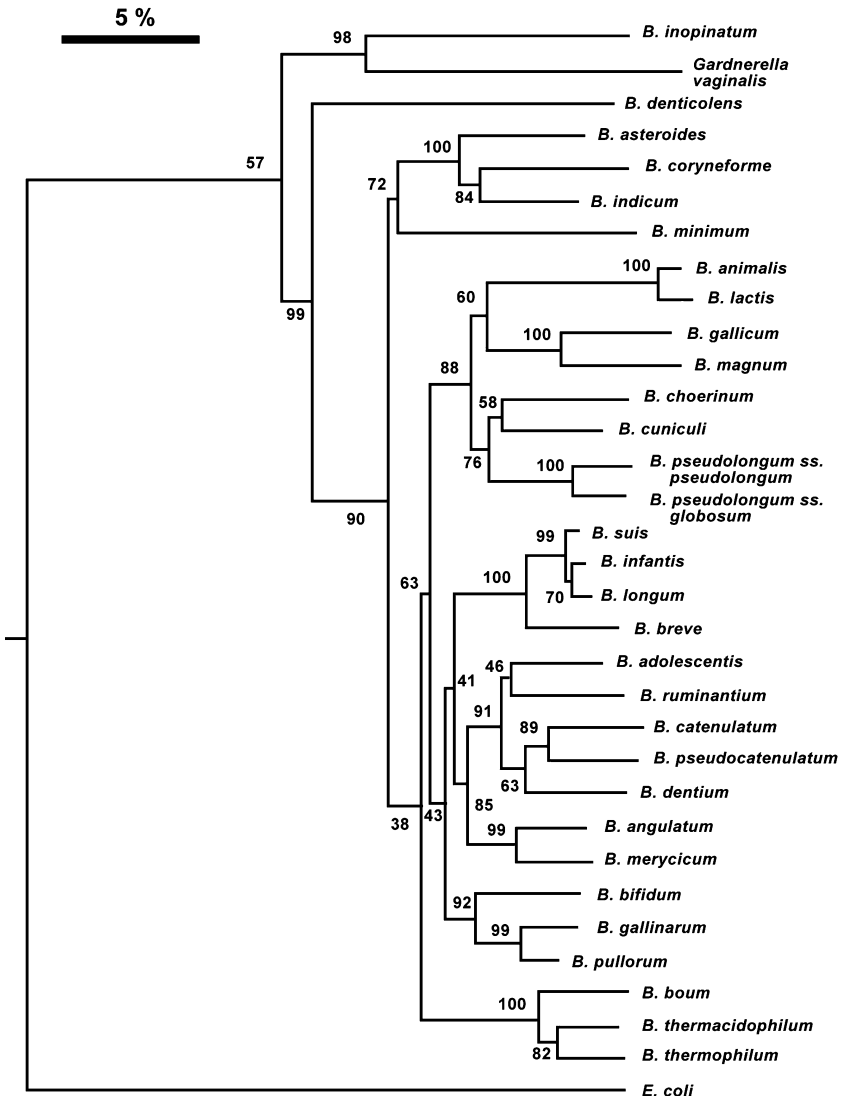

Figure 1. Bifidobacteria phylogenic tree based on partial HSP60 DNA sequences. Bar, 5% sequence divergence. Adapted from Jian *et al.*[119]

Bifidobacteria: survival in the gastrointestinal tract

Several studies have addressed quantification of probiotic survival during gastrointestinal transit.[24] Studies using *B. animalis* DN-173 010 demonstrated the high survival of this strain in the small and large intestines when it is ingested in a fermented dairy product. The results of the main studies carried out to assess strain survival are summarized in Table 2.

In a randomized cross-over study, 12 healthy adults were fed 375 g (125 g, three times a day) of fermented milk containing at least 3.8×10^9 CFU (2×10^7 CFU/g equivalent to 7.5×10^9 CFU) of *B. animalis* DN-173 010. More than 10^8 CFU/g were found in the stools, reflecting the strong survival of that strain during its gastrointestinal transit.[25] Strain survival under exposure to the gastric environment has been shown to be strain-specific both *in vitro* and *in vivo*.[23] *In vitro*, *B. animalis* DN-173 010 and another commercially-available strain, contained in two different fermented dairy products, behaved, indeed, very differently when exposed to a simulated gastric environment; *B. animalis* DN-173 010 survived very well for at least 90 min ($>10^7$ CFU/g), while the other commercial strain was much less resistant (6×10^5 CFU/g).[23] In an *in vivo* study in man, in which gastric fluid specimens were obtained by intubation, the same authors confirmed that the postgastric survival rate of *B. animalis* DN-173 010 was high (80%).[23]

As shown in healthy adults, using intestinal tubing reaching the ileum, 23.5 ± 10.4% of orally administered *B. animalis* DN-173 010 survived during passage

Table 2. Main clinical studies having used *Bifidobacterium animalis* DN-173 010

Subjects	Study design	*B. animalis* DN-173 010 consumption (no. of subjects, quantity, duration)	Method/endpoints	Main results	Reference
Clinical studies on DN-173 010 survival					
Healthy adults aged 17–50 years 6 men/6 women	Randomized, cross-over study	$n = 12$ 375 g of FM containing at least 3.8×10^9 CFU *B. animalis* DN-173 010 vs. Yoghurt 10 days	Stool collection Bacterial count	The quantity of *B. animalis* DN-173 010 surviving digestive tract was $>10^8$ CFU/g *B. animalis* DN-173 010 was still detected 10 days after consumption was stopped	(25)
Healthy young adults	Randomized, cross-over, double-blind study	$n = 12$ 250 g of FM containing at least 2.5×10^9 CFU *B. animalis* DN-173 010 vs. another Bifidus-FM 1 intake	Gastric tube Bacterial count	The quantity of *B. animalis* DN-173 010 surviving gastric transit was $>10^7$ CFU/g	(23)
Healthy adults aged 18–30 years 2 men/4 women	Open study	$n = 6$ 400 g of FM containing about 3.8×10^{10} CFU *B. animalis* DN-173 010 1 intake	Ileal tube and marker of ileal flow rate Bacterial count	The quantity of *B. animalis* DN-173 010 surviving gastric and ileal transit is $>10^7$ CFU/L of ileal fluid. 8 h after ingestion, 23.5 ± 10% of *B. animalis* DN-173 010 is found in terminal ileum	(21)
Healthy women aged 20–48 years	Open study	$n = 5$ 375 g of FM containing about 10^{10} CFU *B. animalis* DN-173 010 7 days	Stool collection Bacterial count associated with colony immunoblotting	The quantity of *B. animalis* DN-173 010 surviving digestive tract is $>10^8$ CFU/g	(26)
Clinical studies on *B. animalis* DN-173 010 effects on transit time					
Healthy adults aged 21–42 years 36 men/36 women	Double-blind, parallel, controlled study	$n = 36$ 375 g of FM containing 9.75×10^{10} CFU *B. animalis* DN-173 010 11 days vs. heat-treated FM	Transit time in colonic segments Radio-opaque pellets	With living *B. animalis* DN-173 010: reduction of total colonic transit time of 21% (men: $P < 0.03$, women: $P < 0.05$). Reduction of sigmoid transit time of 39% ($P = 0.02$), especially in women With heat-treated *B. animalis* DN-173 010: no significant effect on transit time	(30)

Subjects	Study design	*B. animalis* DN-173 010 consumption (no. of subjects, quantity, duration)	Method/endpoints	Main results	Reference
Healthy elderly aged 60–75 years, in two groups: Gr 1 (50 subjects): transit time <40 h Gr 2 (50 subjects): transit time >40 h	Randomized, parallel study	$n = 100$ 250 or 375 g of FM containing 1.25×10^9 to 2.5×10^{10} CFU *B. animalis* DN-173 010 2 weeks	Oro-faecal transit time Coloured marker	Reduction of transit time in all groups compared with baseline ($P < 0.001$). Effect significantly higher with 375 g vs. 250 g of FM ($P < 0.05$) Results among people with transit time <40 h: reduction of transit time of 6.9% with 250 g and 11.8% with 375 g Results among people with transit time >40 h: reduction of transit time of 38.6% with 250 g and 46.4% with 375 g	(33)
Women aged 18–45 years (21 with a long transit time, >40 h)	Randomized, double-blind, cross-over, controlled study	$n = 32$ 375 g of FM containing 3.75×10^9 to 10^{10} CFU *B. animalis* DN-173 010 vs. yoghurt 10 days	Transit time in colonic segments Radio-opaque pellets	Reduction in colonic and sigmoid transit times compared with control ($P < 0.05$). No effects of FM on the faecal concentrations of total secondary bile acids, deoxycholic acid and lithocholic acid	(31)
Healthy elderly aged 50–75 years, in two groups: Gr 1 (100 subjects): transit time = 40–50 h Gr 2 (100 subjects): transit time >50 h	Randomized, parallel study	$n = 200$ 125 or 250 g of FM containing 1.25×10^9 to 2.5×10^{10} CFU *B. animalis* DN-173 010 2 weeks	Oro-faecal transit time Coloured marker	Reduction of transit time in all groups compared with baseline ($P < 0.05$) Effect significantly higher with 250 g vs. 125 g of FM ($P < 0.05$) Results among people with transit time 40–50 h: reduction of transit time of 20.5% with 125 g and 42.2% with 250 g. Effect still significant 2–4 weeks after consumption was stopped ($P < 0.05$) Results among people with transit time >50 h: reduction of transit time of 27.7% with 125 g and 38.1% with 250 g. Effect still significant 2–6 weeks after consumption was stopped ($P < 0.05$)	(32)

CFU, colony forming unit; FM, fermented milk, Gr, group.

through the stomach and small intestine. The strain was recovered at a flow of $10^{8.8}$ CFU/h.[26] Figure 2 shows the survival of bifidobacteria in simulated gastric environment at various pH. More recently, the digestive survival of *B. animalis* DN-173 010 was confirmed in five women aged 20–48 years who consumed 375 g per day of fermented milk for 7 days.[27]

Similar results were reported in studies using other *Bifidobacterium* species.[22, 28] In a study involving eight healthy volunteers, the faecal recovery rate for a variant of *Bifidobacterium* sp. (*B. bifidum*) that could be distinguished from indigenous bacteria was 29.7 ± 6.0% of the ingested dose. When administration of this strain was discontinued, the strain was no longer recovered from the faeces, indicating that *Bifidobacterium* sp. survives in, but does not colonize, the human colon.[22] Similarly, Kullen *et al.* [28] fed a single commercially-available *Bifidobacterium* strain to human volunteers and investigated the faecal bifidobacteria flora using a molecular method. As long as feeding continued, total bifidobacteria (including the administered strain) excretion increased, but the test strain disappeared from the faeces after feeding discontinuation.

These studies clearly show that several bifidobacteria strains, including *B. animalis* DN-173 010, survive gastrointestinal transit without colonizing the gut. Large numbers reach the colon. The high survival rate enables the bacteria to exert physiological effects of potential benefit to the host.

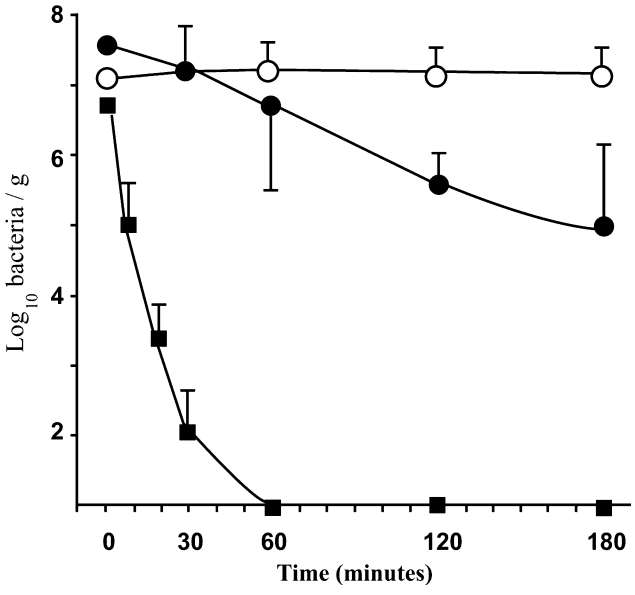

Figure 2. Survival of bifidobacteria after incubation at pH values of 1 (rectangles), 2 (closed circles) or 3 (open circles), as determined by counts of viable bacteria. Mean ± s.d.; $n = 5$ assays. Adapted from Pochart *et al.*[21]

PHYSIOLOGICAL EFFECTS AND CLINICAL BENEFITS OF BIFIDOBACTERIA

The results of the main human and animal studies carried out to further elucidate the physiological effects of *B. animalis* strain DN-173 010 and to assess their clinical pertinence are summarized in Tables 2 and 3.

Transit time

Disturbances of colonic transit, associated with diarrhoea and constipation, are frequent and constitute an important target for functional food, including probiotics.[7] Several bacterial strains have demonstrated activity against diarrhoea of various aetiologies.[29]

However, several studies have evidenced that probiotics accelerate transit time. In a parallel double-blind study including 70 healthy volunteers, the ingestion 375 g/day (125 g, three times a day) of milk fermented by *B. animalis* strain DN-173 010 for 11 days shortened the total colonic transit time by about 20% vs. the baseline colonic transit time and that of placebo group. The effect was more pronounced in women, particularly in those with a long baseline transit time.[30] These beneficial effects were not found with heat-treated probiotics products, suggesting that both probiotic survival and metabolic activity are necessary.[30]

Another double-blind, randomized, controlled study has shown that healthy women had shorter ($P < 0.05$) total colonic and sigmoid transit times following ingestion of 375 g/day of a fermented milk containing yoghurt cultures plus *B. animalis* DN-173 010 for 10 days compared with the time for the *B. animalis* strain-free product.[31] Ingestion of 375 g of product corresponds to ingestion of 3.6×10^{10} CFU of *B. animalis* DN-173 010 which is of the same order of magnitude on the logarithmic scale than the quantity brought by 125 or 250 g of product (1.2×10^{10}, 2.4×10^{10} CFU respectively). While faecal weight, bacterial mass and faecal excretion of secondary bile salts were not significantly influenced, faecal primary bile acid concentrations tended to increase after consumption of the *Bifidobacterium*-fermented milk, an

Table 3. Main animal studies having used *Bifidobacterium animalis* DN-173 010

Subjects	Study design	*B. animalis* DN-173 010 consumption (no. of subjects, quantity, duration)	Method/endpoints	Main results	Reference
Male Sprague–Dawley rats, in four groups fed: 30 g water (Gr 1) 30 g water containing 2.1×10^{10} *B. animalis* DN-173 010 (Gr 2) 30 g FM containing 2.1×10^{10} *B. animalis* DN-173 010 (Gr 3) 27 g uninoculated skim milk (Gr 4)	Experimental diet during 4 weeks followed by two intraperitoneal injections of 1,2-dimethylhydrazine	$n = 28$ 6×10^9 CFU/day (calculated on the basis of mean amount of diets ingested daily) 34 days	Aberrant crypts foci Enzymatic dosages	Reduction of aberrant crypts incidence in groups 2–4 compared with the control diet (− 61% in Gr 2; −49% in Gr 3 and −51% in Gr 4) Reduction of both faecal β-glucuronidase and UDP-glucuronyl-transferase activities in groups 3 and 4 ($P < 0.01$ to $P < 0.05$). Reduction of only faecal β-glucuronidase in Gr 2 ($P < 0.01$) No effects of diets on faecal pH	(108)
Castrated Large White pigs, in two groups receiving either: living bacteria (Gr 1) or killed bacteria (Gr 2)	2 replicate trials (6 pigs/trial)	$n = 6$ 7×10^{11} CFU/day in two daily doses 2 weeks	Extraction and analysis of bile acids from portal serum	None of the treatments modified the portal serum concentration of total bile acids over a 6-h postprandial period Unconjugated bile acids represented up to 44% and 53% of total bile acids, respectively, after 1 and 2 weeks of treatment with living bacteria, vs. only 25% ($P < 0.05$) before treatment or after 1 or 2 weeks of treatment with killed bacteria	(104, 105)
Weaning male F344 rats, in four groups supplemented with: 20% water (Gr 1) 30% non fermented skim milk (Gr 2) 30% *B. animalis* DN-173 010-FM (Gr 3) 30% *S. thermophilus* DN-001 158-FM (Gr 4)	Experimental diet during 1 week followed by HAA consumption during 7–8 weeks	$n = 15$ $5.4 \pm 1 \times 10^8$ CFU/day (calculated on the basis of mean amount of diets ingested daily) 8–16 weeks	Aberrant crypts assessment Enzymatic dosages 3D test Comet assay	Reduction of aberrant crypts incidence compared with the control diet (−66% in Gr 2; −96% in Gr 3 and −93% in Gr 4). Decrease of HAA metabolism, faecal mutagenicity and colon DNA lesions	(83)

Table 3. Continued

Subjects	Study design	*B. animalis* DN-173 010 consumption (no. of subjects, quantity, duration)	Method/endpoints	Main results	Reference
Adult gnotobiotic mice harbouring only the *B. animalis* DN-173 010 strain Adult germ-free mice	Infection with a heterologous simian rotavirus strain (SA-11) Dose: 3×10^8 PFU	$n = 8$ Gnotobiotic mice infected with rotavirus 3 weeks after *B. animalis* DN-173 010 implantation	Stool collection ELISA ELISPOT	The number of total IgA secreting cells are greater in gnotobiotic mice than in germ-free mice ($P = 0.01$)	(60)

CFU, colony forming unit; DMH, dimethylhydrazine; FM, fermented milk; Gr, group; HAA, heterocyclic aromatic amines; PFU, plaque-forming units.

effect which could simply be due to the shorter colonic transit time.[32]

Two studies further investigated the efficacy of different doses of *B. animalis* DN-173 010-containing fermented milk on transit time, by focusing on elderly subjects.[33, 34] The first showed that regular consumption of 250 or 375 g/day of *Bifidobacterium* fermented milk significantly shortened the gut transit time ($P < 0.001$). The effect was more marked with 375 g/day than 250 g/day ($P < 0.05$).[35] A second large-scale and open controlled study evaluated lower doses and the duration of the beneficial effects after consumption of the product has been discontinued. The study included 200 elderly volunteers, aged 50–75 years, divided into two groups – 100 with normal transit time (40–50 h) and 100 with a slow transit time (>50 h) – who were randomized to receive either 125 or 250 g of *Bifidobacterium*-fermented milk daily for 2 weeks.[33] These authors concluded that: (i) in volunteers receiving 125 and 250 g/day *Bifidobacterium*-fermented milk, both dosages significantly reduced oro-faecal transit time. These results are shown in Figure 3. The reduction were 20 and 42% in the group with normal transit time and 28 and 38% in the group with as slow baseline transit time, respectively; and (ii) the effect upon oro-faecal transit time lasted from 2 to 4 weeks after *Bifidobacterium*-fermented milk cessation. These results are shown in Figure 3.

Finally, the data show that milk fermented with probiotic *Bifidobacterium* reduces transit time with a dose–effect response, especially in subjects with slow transit time.

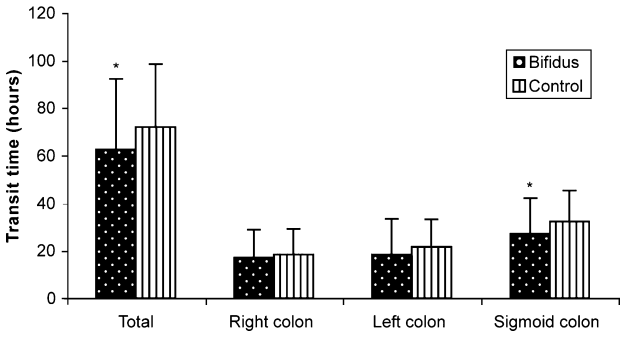

Figure 3. Total and segmental colonic transit time (CTT) at the run-in period and after a 10-day consumption of *Bifidobacterium animalis* DN-173 010-fermented milk. * $P < 0.05$. Adapted from Marteau *et al.*[31]

Colonic fermentation

Through fermentation, bacterial growth is stimulated (biomass), and organic acids (lactic acid and short chain fatty acids-SCFAs), are produced together with gases: H_2, CO_2 and CH_4. Lactic acid is produced by many gut bacterial species, mainly bifidobacteria and lactobacilli. SCFAs (mainly acetate, propionate and butyrate) are the major end-products of bacterial fermentative reactions in the colon and the principal anions in the human hindgut.[34] All SCFAs are rapidly absorbed from the hindgut and stimulate salt and water absorption. They are then metabolized, principally by the gut epithelium, liver and muscle. One of their major properties is their trophic effect on the intestinal epithelium. Moreover, butyrate, a most interesting SCFA, is an important energy source for the colonic epithelium and regulates cell growth and differentiation.[35–37] Even if bifidobacteria do not produce butyrate directly, they produce lactate that may be transformed in butyrate.[38] Butyrate has been shown to reduce the rate of transformed cell growth, in a concentration-dependent manner, and to promote expression of differentiation markers *in vitro*, thus leading to cells reversion from a neoplastic to a non-neoplastic phenotype.[37]

In addition to fermentation products, gut bacteria, including bifidobacteria are able to synthesize vitamins, especially B vitamins.[39, 40] No *in vivo* data concerning the production of B vitamins by bifidobacteria and its impact on B vitamins status in humans is available at the present time.

Barrier effects

A number of mechanisms by which probiotics may protect the host from potentially harmful entities have been proposed, e.g. production of inhibitory substances, blockade of adhesion sites and stimulation of immunity.[41]

Production of inhibitory substances. *Bifidobacterium infantis* strain has been shown to exert a broad spectrum of antimicrobial properties through production of antimicrobial compounds, unrelated to acid production, which inhibit the growth of pathogens.[42] In other studies, the activity of bifidobacteria strains *in vitro* was shown to result from antimicrobial compounds present in the spent culture supernatants, suggesting that the compounds were secreted.[41] Interestingly, Fujiwara *et al.*[43] recently described a protein factor produced by *Bifidobacterium longum* SBT 2928, with a molecular weight of at least 100 000, which inhibited adhesion of enterotoxigenic *Escherichia coli* strain Pb176 which expresses colonization factor adhesion II, to the gangliotetrasylceramide GA1 molecule *in vitro*. Two strains of bifidobacteria were found to produce an antibacterial lipophilic factor (or several factors) with an estimated molecular weight of <3500.[41]

Blockade of adhesion sites. Probiotics may prevent infection by out-competing with pathogenic viruses or bacteria for binding sites on epithelial cells.[44–46] In a study using human Caco-2 cell cultures, *B. animalis* DN-173 010 demonstrated adhesion properties to human cells, even when EGTA was added to the medium: this confirms that adhesion of this *Bifidobacterium* strain to intestinal cells is not calcium-dependent. Further investigation on this strain showed that no extra cellular protein factor is required for its adhesion (A. Servin, personal communication).

Stimulation of immunity. In experimental conditions, *B. longum* increases the immunological and defensive functions of germ free mice.[47–49] *Bifidobacterium breve* YIT4064 enhances antigen specific IgA-antibody directed against rotavirus in the mouse.[50]

The barrier effect generated by some probiotics may derive from positive modulation of the mucous layer that separates the intestinal lumen from the colonocytes. Indeed, probiotics may change the gut mucosal barrier by stabilizing the intestinal mucosa, normalizing intestinal permeability and improving gut immunology, leading to the prevention of the overgrowth of pathogenic bacteria and viruses.[50, 51]

Much work remains to be done to specify the mechanisms of action of particular probiotics against particular pathogens and to show the translation of these mechanisms into human benefits.

Effects on colonic immune system

The first contact that ingested bacteria have with the immune system is the gut-associated lymphoid tissue (GALT).[52] The human intestine is the largest mass of lymphoid tissue in the body, containing over 10^6 lymphocytes/g of tissue. Different components of the mucosal immune system act to focus a specific response against exogenous antigens. The first line in this defence is the secretory IgA system,[52] which produces abundant

mucosal antibodies. The main function of secretory antibodies, in cooperation with non-immunological defence mechanisms, is to mediate exclusion of foreign antigens by preventing epithelial adherence and penetration of invasive pathogenic microorganisms. The antibodies are also responsible for neutralizing toxins and viral multiplication.[53]

The dual role of the digestive flora with respect to the immune is noteworthy. Bacteria (pathogenic or non-pathogenic) constitute antigens that elicit specific systemic and local immune responses. Furthermore, they exert a considerable influence on the number and distribution of the GALT cell populations and play an important role in the regulation of immune responses.[54] These findings mainly derive from animal studies using germ-free and gnotobiotic animal models. Direct evidence in humans is scarce and only hypotheses can be extrapolated from experimental results mainly obtained in mice. In any event, the cellular and molecular events through which the digestive flora modulates the immune system are still poorly understood.[52]

Several probiotics have been reported to stimulate the immune system through non-specific modes of action, resulting in increased immune responsiveness to a wide variety of antigens. In most studies, markers of immune response, rather than disease symptoms, were studied in order to elucidate the mechanisms involved.[55] In uncontrolled studies, *B. bifidum* Bb12 (1×10^{10} CFU/day) and *Lactobacillus acidophilus* La1 (7×10^{10} CFU/day), each given to 14 volunteers for 3 weeks, doubled the number of peripheral white blood cells with phagocytic activity from baseline to the end of the follow-up.[56, 57] In a controlled study, *B. bifidum* and *L. acidophilus* (8×10^{6} CFU/day of each for 28 days) were shown to reduce colonic inflammatory infiltration in 15 elderly subjects.[58] These subjects were 25 institutionalized patients aged >70 years with no overt diseases, according to anamnesis and absence of symptoms such as fever, pain, cough, dysuria and modification of bowel habits. All subjects underwent colonoscopy and multiple endoscopic biopsies, in addition to measurement of blood parameters. The probiotic group showed reduced ($P < 0.02$) total number of T, B and Leu7 lymphocytes per field in the sigmoid and descending colon; peripheral B lymphocytes increased significantly. No colonic or blood changes were seen in the placebo (sucrose and gelatine) group.[58] Further studies using non-enriched fermented milk as a placebo need to be conducted.[59]

Enhancement of the non-specific immune phagocytic activity of granulocyte populations in the blood of human volunteers has been reported following consumption of *L. acidophilus* and *Bifidobacterium* sp.[56] As phagocytic activity contributes to natural immunity and phagocytes are involved in antibody immune responses as antigen-presenting cells, the stimulation of intestinal IgA antibody responses induced by tested bacteria may be partly explained by an effect on phagocytic cell functions. Using ELISA and ELISPOT methods, Moreau *et al.* evaluated the immunostimulating properties in mice of *B. animalis* DN-173 010 in fermented milk by measuring the intestinal IgA anti-rotavirus antibody responses, both in the faeces and in small intestine *lamina propria* cells. Adult gnotobiotic mice harbouring only the *B. animalis* DN-173 010 strain in the gut were infected with a heterologous simian rotavirus strain (SA-11) and the intestinal IgA antirotavirus response compared with that of germ-free mice.[60, 61] The results provided evidence on the adjuvant effect of *B. animalis* DN-173 010 strain on the enhancement of the intestinal anti-rotavirus IgA antibody response at both the cellular and faecal levels.[60, 61]

These data are in line with studies reporting positive effects of probiotics on various gastrointestinal diseases including infant diarrhoea caused by rotavirus infection.

Effects of bifidobacteria on gastrointestinal disease

Infectious diarrhoea. Acute infections of the gut are usually self-limiting and characterized by diarrhoea and, often, vomiting. The principal pathogens are viruses and bacteria. Considering the absence or small number of studies specifically relating to bifidobacteria alone in this section, clinical trials involving mixed preparation of probiotics have been introduced.

Diarrhoea because of rotavirus infection

Rotavirus is the most common cause of acute childhood diarrhoea.

Many clinical studies evaluated the effect of probiotics on rotavirus-associated acute diarrhoea, especially in children. Saavedra *et al.* conducted a double-blind, placebo-controlled trial. Fifty-five hospitalized infants who were randomized to receive a standard infant formula or the same formula supplemented with

B. bifidum (later renamed *B. lactis*) and *Streptococcus thermophilus*.[62] During the 17 months of follow up, 31% of the patients given the standard infant formula, but only 7% of those receiving the probiotic supplemented formula developed diarrhoea. The prevalence of rotavirus shedding was significantly lower in the infants receiving the probiotic supplemented formula.[62] This effect was confirmed in a prospective study including 175 children. The study showed that those receiving bifidobacteria-supplemented milk-based formula were protected against symptomatic rotavirus infection.[63] The prophylactic effect were recently confirmed in a multi-centre, double-blind, controlled trial involving 90 infants aged <8 months who lived in residential nurseries or foster care centres. The study evaluated the efficacy of a milk formula supplemented with viable *B. lactis* strain Bb 12 in terms of the prevention of acute diarrhoea. The number of days with diarrhoea and the daily probability of diarrhoea were significantly reduced in the probiotic group (1.15 ± 2.5 and 0.84 days) vs. the conventional formula group (2.3 ± 4.5 and 1.55 days).[64] Feeding infants with *B. lactis* reduced their risk of contracting diarrhoea 1.9-fold (range, 1.33–2.6).[65]

Antibiotic-associated diarrhoea. Diarrhoea caused by the growth of pathogenic bacteria is the most common side effect of antibiotic use. Probiotics may inhibit this growth by releasing inhibitory substances or bacteriocins, as has been demonstrated with some strains *in vitro*.[59, 66, 67] To date, the main probiotics used are *Lactobacillus* GG, *Enterococcus* SF68 and *Saccharomyces boulardii*.[68] One double-blind placebo-controlled study of 10 adults tested the effects of a daily consumption of 3 cups/day of *B. longum* yoghurts on erythromycin-associated gastrointestinal effects.[69] Faecal weight, stool frequency, and abdominal complaints were significantly increased when erythromycin was given with placebo yoghurt but not when *B. longum* yoghurts were being taken. Moreover the simultaneous intake of *B. longum* yoghurts with erythromycin induced a sharp fall in clostridia spore count, suggesting that these yoghurts could reduce antibiotic-associated alterations in the intestinal microflora. In another study, subjects receiving a mix of prebiotics (fructooligosaccharides) and probiotics (including *B. longum* BB 536) during oral administration of cefpodoxime proxetil twice daily were shown to be less susceptible to *Clostridium difficile* colonization than subjects receiving prebiotics only or placebo.[69] These results were confirmed in a recent double-blind, placebo-controlled study investigating the role of a probiotic containing both *Lactobacillus* and *Bifidobacterium* in the prevention of *C. difficile*-associated diarrhoea. The study was conducted on 150 elderly patients receiving antibiotic therapy and randomized to receive the treatment for 20 days. For the patients developing diarrhoea, the incidence of samples positive for *C. difficile*-associated toxins was 2.9% in the probiotic group vs. 7.25% in the placebo-control group. When specimens from all patients were tested, 46% of probiotic patients were *C. difficile* toxin-positive vs. 78% in the placebo group.[70]

Pouchitis and human inflammatory bowel disease. Acute or chronic inflammation occurs in up to 50% of ulcerative colitis (UC) patients following proctocolectomy and pouch reconstruction associated with ileoanal anastomosis.[71] Available data on probiotics and pouchitis are obtained with mixed strains mostly *Lactobacillus* and bifidobacteria. Much work remains be done to investigate the role for bifidobacteria in this disease.

The most convincing evidence of the clinical effect of probiotics in human inflammatory bowel diseases[72] was generated by a small prospective, double-blind, placebo-controlled trial showing that a combination of eight probiotic bacteria including three strains of bifidobacteria (*longum*, *breve* and *infantis*) prevented relapse of chronic pouchitis after induction of remission by antibiotics.[73] These results have been replicated[74] and partly extended by probiotic administration immediately after ileostomy closure.[75] Similar favourable clinical results of a mixture of lactobacilli (La-5) and bifidobacteria (Bb-12) on symptoms and endoscopic inflammation in UC patients with pouchitis have been reported.[76] Other evidence, came from two studies from Ishikawa *et al.*[77] and Kato *et al.*[78] showing the effectiveness of supplementation with bifidobacteria-fermented milk containing live bifidobacteria (*breve* and *bifidum*) and *L. acidophilus* YIT 0168) in the treatment of UC. Both studies were randomized controlled trials – one being placebo-controlled[78] – but one deals with mild to moderate, active UC [78] and the other with the maintenance of remission in UC.[77] In a recent randomized controlled pilot trial, the short-term synbiotic treatment of active UC was shown to improve the full clinical appearance of chronic inflammation in patients receiving this therapy.[79] The precise role of each probiotic species,

including bifidobacteria, and prebiotic substances in these results remains to be determined.

Irritable bowel syndrome. While most, if not all, of 12 clinical trials of probiotics in irritable bowel syndrome (IBS) have concerned lactobacilli (mainly *L. plantarum* and *Lactobacillus* GG) and mixtures of lactobacilli and bifidobacteria strains,[80] superiority for bifidobacterium (*B. infantis* 35624) over both a lactobacillus (*L. salivarius* UCC4331) and placebo for alleviating each of the cardinal symptoms of IBS (except for bowel movement frequency and consistency) and for a composite score has been very recently shown.[81]

Colonic tumours. Among environmental factors, genotoxic chemicals ingested in diet may be involved in the development of colorectal cancer, a significant cause of mortality in Western industrialized countries.[82] Some chemicals are thought to induce the initiation step of the carcinogenetic process, while the majority of them are involved in the promotion step.[83]

Bifidobacteria, bacterial metabolism and colonic carcinogenesis

Evidence is accumulating that the normal intestinal flora can influence carcinogenesis by producing enzymes that transform procarcinogens into active carcinogens. These enzymes include β-glucuronidase, azoreductase and nitroreductase.[84–88]

Bacterial β-glucuronidase in the colon is able to release carcinogens from hepatic-derived glucuronic acid conjugates and is a critical factor in the enterohepatic circulation of drugs and other foreign compounds. As mentioned by Rafter *et al.*: 'Although it represents a simple reproducible marker, evidence for a role for β-glucuronidase in human colorectal cancer is indirect and is remote from the final end-point (tumours)'.[89]

Azo- and nitroreductases reduce their substrates to amines, which are usually more toxic than the parent compound, and nitrate reductase generates the highly reactive and toxic anion, nitrite.[90] Ammonia is considered to be a potential tumour promoter in the colon, and the hypothesis that it enhances neoplastic transformation in the gut has been advanced. Other gut bacterial products with possible adverse effects on the colonic mucosa include secondary bile acids, which are potential harmful substances.[71] They may exhibit carcinogenicity by acting on the mucous-secreting cells and promoting their proliferation, or they may act as promoters of carcinogenesis.[91]

There is some evidence that selected microorganisms, such as probiotic bifidobacteria, may protect the host from carcinogenic activity by decreasing the production and/or activity of these potential carcinogens.[92–94] There is experimental date to suggest that probiotic metabolism may indeed beneficially influence faecal enzymes activity.[91] For example, consumption of milk fermented with a *Bifidobacterium* species for 12 days decreased β-glucuronidase activity compared with baseline, even though if it had no effect on faecal pH or the activity of nitrate reductase, nitroreductase and azoreductase.[95] In another study, consumption of a fermented milk with *L. acidophilus*, *B. bifidum*, *Streptococcus lactis* and *Streptococcus cremoris* for 3 weeks decreased the activity of nitroreductase from baseline, even though if it did not modulate the activity of β-glucuronidase and azoreductase.[87] *In vitro* and *in vivo* in rats, *B. animalis* DN-173 010 shows β-fructofuranosidase and β-galactosidase activities. These activities are enhanced by some prebiotics like transgalactooligosaccharides (C. Andrieux, personal communication). β-Fructofuranosidase and β-galactosidase are considered as positive markers of colon health.[96]

Bifidobacteria, nitrosamines, nitrites and heterocyclic amines

Positive modulation of nitrosamines, nitrites and heterocyclic amines production by bifidobacteria has been reported.[97, 98] An *in vitro* study showed that the growth of bifidobacteria strains was not affected by low nitrite concentrations and that acids produced by bifidobacteria seemed to be involved in nitrite elimination.[99] With regards to heterocyclic amines, anti-mutagenic effects were demonstrated in a study investigating the efficacy of a wide range of LAB against 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) mutagenicity; a significant anti-mutagenic effect was shown with eight *Lactobacillus* out of 76 LAB.[98] Other bacteria were studied, using TNO's *in vitro* large intestine model (TIM-2). In this model, the potential beneficial effect of *B. animalis* DN-173 010 on bioconversion and mutagenicity of heterocyclic amines was confirmed. This finding has been reported at Congrilait in 2002 (K. Venema, personal communication).

Bifidobacteria and their effects on bile acids

Many intestinal bacteria, including *Bifidobacterium* and *Lactobacillus* species, can deconjugate or hydrolyse conjugated bile acids.[100] Between-species differences in hydrolase activity has been evidenced.[101]

Although initially considered as useful property,[102] this activity has since been suggested to constitute a health disadvantage as it may increase the formation of secondary cytotoxic bile acids.[100, 103] However, a recent trial performed in pigs, receiving either living bacteria or killed bacteria for 2 weeks, showed that *B. animalis* DN-173 010, which has a bile salt hydrolase activity *in vitro*, was also active *in vivo* during its transit through the gastrointestinal tract. Moreover this probiotic strain did not induce cytotoxic bile acids production: unconjugated bile acids levels were significantly lower in the group receiving living bacteria.[104, 105]

In man, a randomized double-blind, controlled trial showed that faecal concentrations of total secondary bile acids, deoxycholic acid and lithocholic acid were not significantly different in healthy women consuming a fermented milk procuring 2×10^{10} to 4×10^{10} CFU/day of *B. animalis* DN-173 010.[106]

Effects of bifidobacteria on cell proliferation

Probiotic-enriched fermented milks may exert beneficial effects on intestinal cell proliferation. Markers such as DNA damage, microadenomas and aberrant crypt foci in the mucosa, can be used to identify early epithelial events linked to colonic cancer. Induction of aberrant crypt foci has been particularly widely used, as it is easily observed macroscopically. In man, aberrant crypts and microadenomas, similar to those described in animals, have been described,[107] but need to be correlated with other well-known markers of tumour risk.

An *in vitro* study using IEC-6 cell cultures demonstrated that fermented milks containing probiotic bifidobacteria stimulate mitochondrial deshydrogenase response, DNA synthesis and cyclic AMP production.[108]

In a pathogenic context, *in vitro* and *in vivo* studies showed that several LAB present in fermented milks may have an inhibitory effect on the development of precancerous lesions and tumours in animal models.[103] In Ames' test, *B. animalis* DN-173 010 has been shown to have an inhibitory effect towards indirect mutagenic agents *in vitro*.[109–111] *In vivo*, in rats, several studies

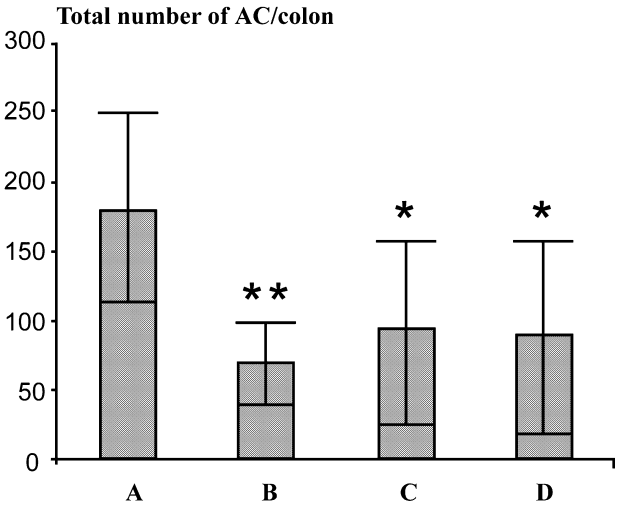

Figure 4. Effect of dairy-supplemented diets on the number of aberrant crypts (AC) in 1,2-dimethylhydrazine-treated rats. A: control diet; B: diet supplemented with DN-173 010 suspension; C: diet supplemented with fermented skim milk by DN-173 010 strain; D; diet supplemented with uninoculated skim milk. Student's *t*-test was used for statistical comparison; ** $P < 0.01$; * $P < 0.05$. Adapted from Abdelali *et al.*[108]

have confirmed the protective effects of milk, fermented milk and various LAB with respect to chemically-induced colonic carcinogenesis.[109, 112, 113] Using aberrant crypts as an oncogenesis marker, Abdelali *et al.* reported a 61% reduction in crypt foci in rats fed a normal diet supplemented with a suspension containing 2.1×10^{10} *B. animalis* DN-173 010 vs. the controls; β-glucuronidase activity was also significantly decreased.[109] Figure 4 illustrates these results.

The above studies mainly used 1,2-dimethylhydrazine or azoxymethanol, its metabolite, as carcinogens and aberrant colon crypts as carcinogenesis markers. Using the comet assay, two short-term studies in rats showed that bacteria such as *Lactobacillus*, *Bifidobacterium* and *S. thermophilus* were able to decrease colon DNA damage after exposure to the genotoxic agent, 1,2-dimethylhydrazine.[113, 114] Using F344 male rats fed a diet supplemented with *B. longum*, the following were demonstrated: (i) a 100% colonic tumour inhibition [115]; (ii) a significant decrease in the number of azoxymethane-induced colonic aberrant crypt foci and in the total number of aberrant crypts [111]; and (iii) a significant suppression of colon tumour incidence, tumour multiplicity and tumour volume.[116] The latter study, also evidenced modulation of the intermediate biomarkers of colon carcinogenesis, such as colonic mucosal and/or

tumour cell proliferation, ornithine decarboxylase activity and ras-p21 oncoprotein expression.[116] A significant decrease in the faecal bacterial β-glucuronidase activity was also observed in the animals fed *Bifidobacterium*-supplemented diets vs. the control diet.[117]

A further carcinogenesis model (heterocyclic aromatic amines) was used to test the preventive potential of fermented milk containing *B. animalis* DN-173 010 and *S. thermophilus* DN-001 158.[83] The fermented milk significantly reduced the total number of aberrant crypts induced by diet containing heterocyclic aromatic amine carcinogens, but non-fermented milk has almost the same effect.[83] These results concord with those of a previous study, which showed that skimmed milk alone decreased the incidence and number of aberrant colonic crypt formation in 1,2-dimethylhydrazine treated rats.[108] In conclusion, the intermediate biomarkers used in the above studies showed that dairy products decreased aberrant colonic crypt formation, which may be operative at the initiation stage of the carcinogenetic process.

Using cultured human colonic cancer cell line HT-29, Baricault *et al.* studied at the cellular level the effect of fermented milks on colon cancer cell growth and differentiation characteristics[118]; *Bifidobacterium* was among the most effective bacterial species that lowered the HT-29 growth rate. Concomitantly, the specific activities of dipeptidyl peptidase IV, a sensitive and specific marker of HT-29 cell differentiation, and that of three other brush border enzymes (sucrase, aminopeptidase N and alkaline phosphatase) were significantly increased, thus suggesting that the cells may have entered a differentiation process.[118]

Overall, these results suggest that probiotic-containing dairy products could help to prevent colonic carcinogenesis. Obviously, more extensive investigations and clinical trials must be conducted on this ongoing topic.

CONCLUSIONS

Bifidobacteria, as probiotics, may become an important means of enhancing digestive health and preventing disease. In order to realize this potential fully, research must focus on the following areas: (i) identification of *Bifidobacterium* strains that can withstand gastrointestinal transit (i.e. gastric acidity, bile salts and Paneth cell secretions); (ii) identification of the *Bifidobacterium* species and strains that are effective against specific disease processes or in disease prevention; (iii) investigation of the mechanisms of probiotic action; and (iv) development of new association between bifidobacteria strains and prebiotics. Currently, the utilization of probiotics and prebiotics is an interesting field of research as several probiotic strains, including *B. animalis* DN-173 010, show a more preferential fermentation pattern when associated with short-chain oligomers than with monomers.[119–121] A recent study[79] has shown a beneficial effect of a prebiotic and probiotic association highlighting the growing interest of synbiotics in digestive health.

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