

Original Article

Isolation of *Bifidobacteria infantis* and its antagonistic activity against ETEC 0157 and *Salmonella typhimurium* S-285 in weaning foods

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Probiotic organism *Bifidobacteria* was isolated from the faeces of breast-fed infants at Universiti Putra Malaysia. Trypticase phytone peptone yeast extract agar (TPY) was used as a selective media for the isolation. Morphological examination of the isolates indicated that *Bifidobacteria* was Gram-positive rods in nature, curved with characteristics of V and Y shapes. The organisms were non-catalase producing, non-nitrate reducing, non-motile, had an absence of indole and were unable to liquify gelatin. The ratios of acetic and lactic acids were determined using high performance liquid chromatography (HPLC). Using carbohydrate fermentation profile test API-CH-50 kits, 20 *Bifidobacteria* strains had been identified: they were the species of *Bifidobacteria infantis* and two different sub-species, mainly *infantis* and *lacentis*. Based on a wide zone of inhibition, three suitable strains of *B. infantis*, Bifi-11, Bifi-19 and Bifi-20, were tested in weaning foods for antimicrobial activity towards two human pathogens: *E. coli*-0157 (World Health Organization) and *Salmonella typhimurium* S-285. The pH, titratable acidity of weaning foods and total colony count for *Bifidobacteria*, enteropathogenic *Escherichia coli* and *S. typhimurium* were recorded at 3-h intervals for 30 h. It was found that after 9 h of incubation of weaning foods, the pH declined to < 3.6 from pH 6.0, whereas titratable acidity increased from 0.026 to 0.08%. It was indicated that *Bifidobacteria* inhibited *E. coli* better than did *S. typhimurium* due to low pH. After 24 h of incubation, approximately 98% of *E. coli* was inhibited by *Bifidobacteria*. It is suggested that the inhibitory effect of *Bifidobacteria* strains in weaning foods towards the growth of enteropathogenic *E. coli* and *S. typhimurium* was solely due to low pH and the production of volatile acid components by the organism.

Key words: antagonistic, *Bifidobacteria infantis*, pathogen, probiotic, *Salmonella typhimurium*, weaning food.

Introduction

Probiotic can be described as organisms and substances that contribute to intestinal microbial balance.¹ Probiotic is 'a live microbial feed supplement which beneficially affects the host animal by improving intestinal microbial balance.'²

The human large intestine can be described as a complex ecosystem. It is thought that at least 50 genera of bacteria reside in the colon, comprising several hundred species.³ The major health-promoting bacteria found in the human gut are the colonic bacteria belonging to the genera *Bifidobacterium*. Studies have shown that this bacteria may have some health-promoting properties, and that their levels can fluctuate and be manipulated.⁴ Attempts have been made to increase the number and activity of these bacteria by consuming live cultures of selected species of bacteria in milk, yogurt or pure cultures in powdered, tablet or capsule form. The potential health benefits of this bacteria during colonization in the large intestine is that it suppressed the undesirable pathogenic organisms, which cause diarrhoeal diseases; inhibited the growth of putrefactive bacteria, which cause liver damage; broke down nitrosamine, the source of carcinogenesis; stimulated immunity systems; and improved digestion and absorption of essential nutrients and synthesis of vitamins.⁵

The concept of weaning refers to a period during which an infant gradually progresses from a milk only diet to one of the foods usually consumed by the family. Traditionally, weaning foods are introduced between the ages 4 and 6 months in order to meet the nutritional requirements of the growing infant.⁶ Breast milk has anti-infective properties, which are both humoral and cellular; protective factors such as immunoglobulins, lysozyme and bifidus factor; and lactoferrin, which limits the availability of iron for intestinal bacteria, especially *Escherichia coli*.⁷ The faeces of breast-fed infants contain higher numbers of *Bifidobacteria* than do the faeces of bottle-fed infants and the numbers of Gram-negative bacteria in the intestines of bottle-fed infants were higher than in breast-fed infants.⁸

The ability of probiotic organisms such as *Bifidobacteria* to produce organic acids such as acetic and lactic acid can

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Accepted 29 October 1999

thus lower intestinal pH and hence inhibit the growth of these pathogenic microorganisms.⁹ Some species of *Bifidobacteria* even produce antibiotic- and bacteriocin-like substances which have antagonistic activity towards certain pathogenic organisms.^{10,11} The majority of these probiotic bacteria improve the intestinal flora, prevent colonization of pathogens, ameliorate diarrhoea and constipation and improve the immune system.¹² They participate in the formation of the mucosa-associated flora in the colon where the microorganisms both adhere to the host cells and embed in the mucin layer and act as a barrier to the pathogenic organisms.^{13,14}

The aim of this study was to isolate the probiotic organisms, such as *Bifidobacteria*, from the faeces of breast-fed infants and to determine their inhibitory activity against the enteropathogenic *E. coli* and *Salmonella typhimurium* in weaning foods. A suitable method for a longer shelf life for weaning foods supplemented with the *Bifidobacteria infantis* species was also studied. Preventive measures could be taken during the diarrhoeal episodes if probiotic weaning foods could be fed to the diarrhoeal infected-infants. Apart from playing an important role in inhibiting the growth of pathogenic organisms, these probiotic weaning foods could also improve the physical and nutritional status of the affected infants.

Materials and methods

Three species of *B. infantis* (Bifi-11, Bifi-19 and Bifi-20) were isolated from the faeces of breast-fed infants aged between 3 and 6 months at Universiti Putra Malaysia. A faeces sample of 1 g was diluted in 9 mL of phosphate buffered saline (PBS; sodium chloride 0.85 g, dibasic sodium phosphate 0.25 g, monobasic sodium phosphate 0.56 g) and 100 mL de-ionized water, final pH of the entire solution was pH 7.0.¹⁵ The sample was homogenized for 30 s on a vortex. Appropriate dilutions were assayed with 0.1% peptone water (Oxoid Unipath; Basingstoke, UK) and plated on a Trypticase Peptone Yeast Extract agar (TPY) recommended by Scardovi¹⁶ and supplemented with 0.5% propionic acid.¹⁷ Plates were incubated at 37°C for 48 h in anaerobic conditions using a gas generating kit (BBL, Dickinson & Co; MD, USA). Colonies were screened by Gram stain morphology. Biochemical identification by fermentation and gas production of glucose by *Bifidobacteria* were performed in sterile tubes containing 5 mL of basal medium, based on the procedure by Macfauddin.¹⁸ The tests for catalase, nitrate reduction, indole formation and gelatin liquifaction were performed according to the *Anaerobic Laboratory Manual*.¹⁹

The ratio of acetic and lactic acid produced by *Bifidobacteria* was determined by high-performance liquid chromatography (HPLC).^{20,21} The column used was Bio-Rad (Bio-Rad Labs; CA, USA) Aminex HPX-87H anion 300 mm × 7.8 mm; sulphuric acid (0.009 N) as the mobile phase was used. The Shimadzu LC-10 as a liquid chromatography with an SPD 210 Av UV-VIS detector set at 210 nm and the flow rate was maintained at 0.7 mL/min. Using a Hamilton syringe, a 20 µL sample was injected into the HPLC. The genus and species of *Bifidobacteria* were identified by their ability to ferment carbohydrate profiles using API-50-CH kits following the method of Scardovi.¹⁶ Based on the antagonistic activity of *Bifidobacteria* towards

the enteropathogenic *E. coli* and *S. typhimurium*, three species of *B. infantis*, subspecies *infantis* and *lacentis*, were selected for the analyses.

Collection of Salmonella and E. coli strains

Freeze drying cultures of *E. coli* ETEC 0157 (World Health Organization) and *Salmonella typhimurium* S-285 were collected from the Culture Collection Center, Institute of Medical Research (IMR), Kuala Lumpur, Malaysia. The *E. coli* and *Salmonella* cultures were maintained by routine culture on a nutrient agar (Difco Laboratories; MI, USA) slant and stored at 4°C between transfer. At least two additional subcultures (24 h, 37°C) were made in a fresh medium before being used in the experiment.

Assay for detection antagonisms of Bifidobacteria

Bacterial antagonisms were tested by a method modified from Francoise *et al.*¹⁰ Trypticase peptone yeast extract agar (TPY; 30 mL) was poured into standard size disposable Petri dishes. The medium was left to solidify and dry under UV light in a laminar flow cabinet for 15 min. Overnight cultures of *Bifidobacteria* were spotted at three points on the surface of the agar with a sterile paper disc. The plates were incubated anaerobically (BBL Gaspak System; Becton Dickison, MD, USA) at 37°C for 24 h. The plates were then overlaid with soft TSA agar (1% agar added to TSB) seeded with 1% freshly prepared *E. coli* and *Salmonella* cultures at 10⁻⁷ concentration. The overlaid cultures were incubated at 37°C for 24 h. The diameter of the zone of inhibition was measured using vernier slide calipers. The diameter of the zones of inhibition was the difference between the total inhibition zone and the diameter of the colony.

Preparation of weaning food

Weaning foods were prepared according to Khaleque and Roy using rice powder (20 g), green gram (15 g), sugar (12 g), β-carotene rich DGLV powder (leaves of dark green vegetables, spinach, colocasia and carrot; 5 g), amylase powder (2 g), energy (1084 kJ) and protein (5.5 g).²² All of the ingredients were washed with distilled water and dried at 60°C for 30 min in a dryer, and ground into a powdered form. The powdered ingredients were mixed together and total weight was 171.21 g. The powdered weaning food was dissolved in 1 L de-ionized water and boiled for 15 min at 100°C. Following this, 200 mL was poured into each of five sterile reagent bottles. The weaning food was autoclaved at 121°C for 15 min and kept in a water bath at 45°C for further analyses.

Microbiological assay of weaning foods

One percent of the overnight culture of *Bifidobacteria infantis* (the optical density at 600 nmol/L is 1.50) was added to 200 mL of the weaning food, followed by 1% of an overnight broth culture of ETEC 0157 *E. coli* or *S. typhimurium* at 10⁻⁶ concentration; these were then mixed homogeneously. At the same time, 1% of a broth culture of ETEC *E. coli* 0157 and *S. typhimurium* were mixed separately in two different 200 mL samples of the weaning food, and these were maintained as the control sample. The inoculated samples of weaning foods were incubated in a water bath at 35°C for 30 h. At every 3-h interval the pH, titratable acidity and total

plate count for *Bifidobacteria*, *E. coli* and *Salmonella* were analysed. For the enumeration of *Bifidobacteria*, trypticase peptone yeast extract agar (TPY; Scardovi¹⁶, 1986) was used for the analyses. Similarly for the growth of *E. coli* and *Salmonella*, nutrient agar (Difco Laboratories; MI, USA) was used. The total colony count for all of these organisms was assayed by spreading 0.1% of a serial dilution on the respective agar media for the growth of *Bifidobacteria*, *E. coli* and *Salmonella*. The TPY plates for *Bifidobacteria* were incubated anaerobically at 37°C for 48 h while the plates for *E. coli* and *Salmonella* were incubated aerobically at 37°C for 24 h. Random colonies were picked up from three different strains and confirmed by Gram staining of morphological analyses. Total colony counts for *Bifidobacteria*, *E. coli* and *Salmonella* were recorded from the growth of the respective agar media.

Statistical analysis

Log colony forming unit for three *B. infantis* species, Bifi-11, Bifi-19 and Bifi-20, and their inhibition towards *E. coli* and *S. typhimurium* were analysed using the statistical software package SAS (Microsoft Windows 97; Microsoft, Seattle, WA, USA) in order to find the significant effect of incubation of three *Bifidobacteria* species with *E. coli* and *Salmonella*.

Results and discussion

Twenty *Bifidobacteria* strains were identified based on their morphological characteristics. Colony morphology can be used for the tentative identification for the genus *Bifidobacterium*.⁴ The analysis of HPLC showed that most of the strains produced acetic and lactic acids from the fermentation of glucose.²³ *Bifidobacteria* species mainly has been identified on the basis of phenotypic characteristics such as carbohydrate fermentation patterns and cellular morphology.²⁴ The biochemical test and fermentation of carbohydrate profile showed the genus and species level of *Bifidobacteria*.²³ Based on the antagonistic activity, three *B. infantis* strains were selected for the analyses. The present study indicates that three species of *B. infantis*, ssp. *infantis* strains (Bifi-11, Bifi-19 and Bifi-20), were mixed with weaning foods and that after 3 hours incubation all of these *Bifidobacterial* species were antagonistic to enteropathogenic *E. coli* and their counts had been drastically reduced (Figs 1–3). Further studies showed that after 3 hours of incubation, species Bifi-19 and Bifi-20 inhibited *E. coli* better than did the species Bifi-11 (Figs 1–3). *Bifidobacteria infantis* species Bifi-11 reduced the population of *E. coli* from 5.65 to 2.0 log c.f.u./g after 30 h of incubation (Fig. 1). Similarly, Bifi-19, and Bifi-20 *B. infantis* species reduced *E. coli* count from 5.92 to 1.0 and from 5.69 to 1.0 log c.f.u./g after 30 h of incubation (Figs 2,3). Results in Fig. 4 showed that the pH and titratable acidity for *B. infantis* Bifi-19 and Bifi-20 species had reduced the total pH from 5.60 to 4.80 after 3 h of incubation and increasing the amount of titratable acidity from 0.05 to 0.09% when compared with Bifi-11 species. This is due to the organic acids produced by *Bifidobacteria* from the fermentation of carbohydrate, which reduced the pH of weaning foods.

Other studies also showed the production of organic acids for Bifi-19 and Bifi-20 *B. infantis* species. It was found that *B. infantis* Bifi-11 species (Fig. 5) reduced the population of *S. typhimurium* from 6.60 to 2.59 c.f.u./g after 30 h of

incubation. Similarly, Bifi-19 and Bifi-20 *infantis* species reduced the number of *S. typhimurium* from 6.53 to 2.44 c.f.u./g and from 6.40 to 2.42 c.f.u./g, respectively, after 30 h of incubation (Figs 6,7). Figure 8 shows the pH and titratable acidity for *B. infantis* species against *S. typhimurium*. It was found that Bifi-19 and Bifi-20 species reduced pH from 6.1 to 5.4 after 3 h of incubation and increased the amount of titratable acidity from 0.04 to 0.07% when compared with Bifi-11 species.

In this study, Bifi-19 and Bifi-20 *infantis* species exhibited maximum antagonistic activity towards *E. coli* and *S. typhimurium* as compared with the Bifi-11 species. *In vitro*

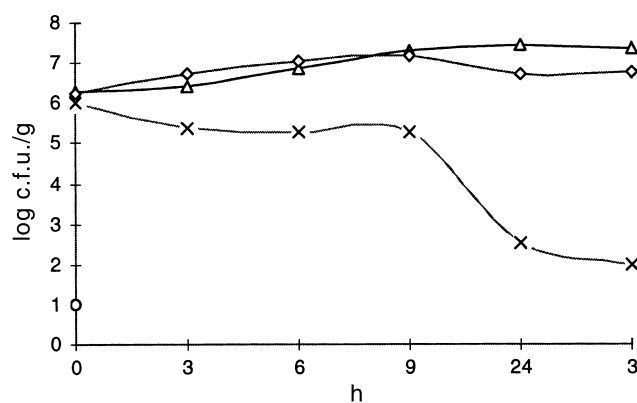


Figure 1. Inhibition of *Escherichia coli* in weaning foods by *Bifidobacteria* (Bifi 11). (Δ) Bifi-11; (\times) Bifi-11 + *E. coli*; (\diamond) *E. coli* control.

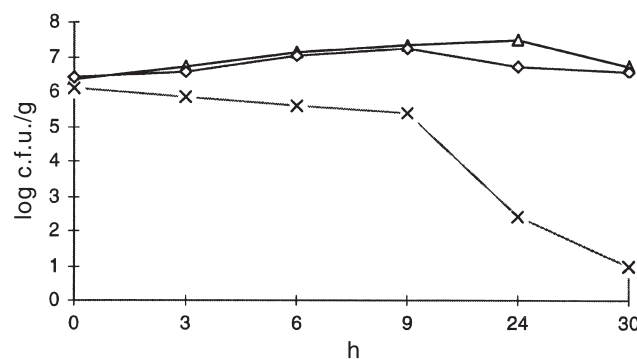


Figure 2. Inhibition of *Escherichia coli* in weaning foods by *Bifidobacteria* (Bifi 19). (Δ) Bifi-19; (\times) Bifi-19 + *E. coli*; (\diamond) *E. coli* control.

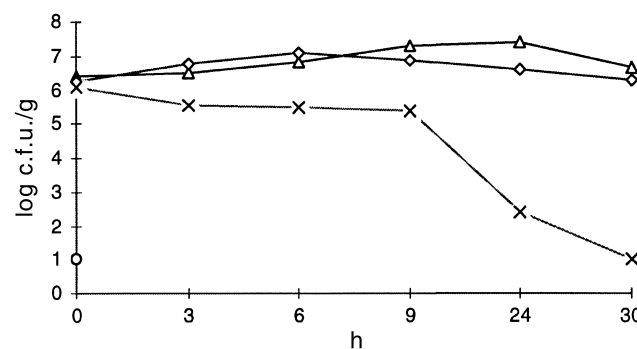


Figure 3. Inhibition of *Escherichia coli* in weaning foods by *Bifidobacteria* (Bifi 20). (Δ) Bifi-20; (\times) Bifi-20 + *E. coli*; (\diamond) *E. coli* control.

studies showed the antagonistic activity of different *Bifidobacteria* strains against *E. coli* and *S. typhimurium*. It can be seen in Table 1 that the antagonistic activity of *E. coli* 0157 for the Bifi-19 and Bifi-20 species had a 17.5 mm and 19.0 mm wide zone inhibition, respectively, while Bifi-11 had 17.0 mm. Similarly for the Bifi-19 and Bifi-20 *infantis* species against *S. typhimurium*, S-285 had a 16.5 mm and 18.0 mm zone of inhibition against *E. coli* and *Salmonella*,

while Bifi-11 had a 16.5 mm zone of inhibition against *E. coli* and *Salmonella*. The inhibitory spectrum of the Bifi-20 species was wider in comparison to the Bifi-19 and Bifi-11 species.

Based on the antagonistic activity *in vitro*, three *Bifidobacteria* strains, Bifi-11, Bifi-19 and Bifi-20, were selected for these analysis. The selection was based on the production of volatile acids by the organisms, which reduced the pH value of weaning foods and increased the percentage of acidity. Also, from the HPLC analysis, it was found that all of these *Bifidobacteria* strains produced acetic and lactic acids from the carbohydrate fermentation.²³ The production of organic acids by *Bifidobacteria* had much more inhibitory activity towards the Gram-negative bacteria *E. coli*, *S. typhimurium* and *Shigella sonnei*.²⁵

Acetic acid is a more effective antimicrobial agent than lactic acid.¹³ The antimicrobial effect of the organic acids is thus principally produced by the undissociated molecules through the acidification of cytoplasm, destruction of the transmembrane of the proton motive force, and loss of active transport of nutrients through the membrane. Acetic acid produced by *Bifidobacteria* was highly lipophilic and able to neutralize transmembrane pH gradients efficiently. Thus, lactic acid, with its lower pK value, has less effect on neutralizing the proton motive force than acetic acid.²⁶ The mechanisms of action of these organic acids are most likely based

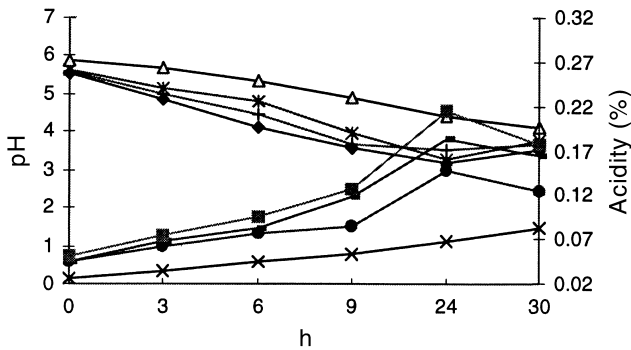


Figure 4. pH and TA in weaning foods by *Bifidobacteria* and *Escherichia coli*. (★) pH : Bifi-11; (●) TA : Bifi-11; (◆) pH : Bifi-20; (■) TA : Bifi-20; (+) pH : Bifi-19; (-■) TA : Bifi-19; (△) pH : *E. coli* control; (×) TA : *E. coli* control.

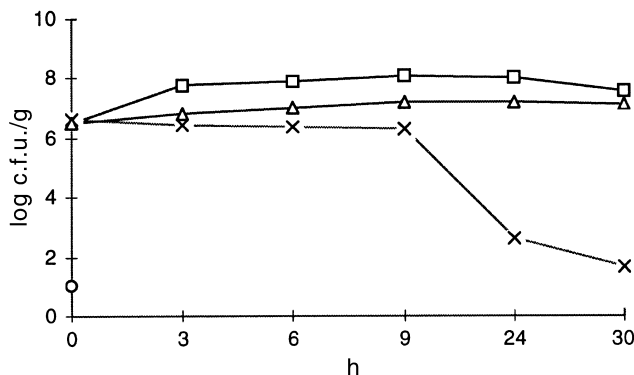


Figure 5. Inhibition of *Salmonella typhimurium* in weaning foods by *Bifidobacteria* (Bifi 11). (□) Bifi-11; (×) Bifi-11 *Salmonella*; (△) *Salmonella* control.

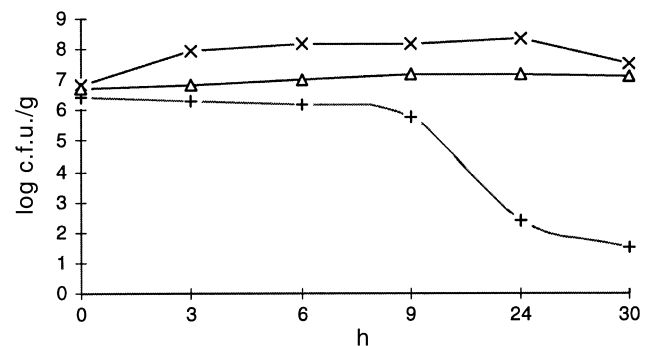


Figure 7. Inhibition of *Salmonella typhimurium* in weaning foods by *Bifidobacteria* (Bifi 20). (×) Bifi-20; (+) Bifi-20 + *Salmonella*; (△) *Salmonella* control.

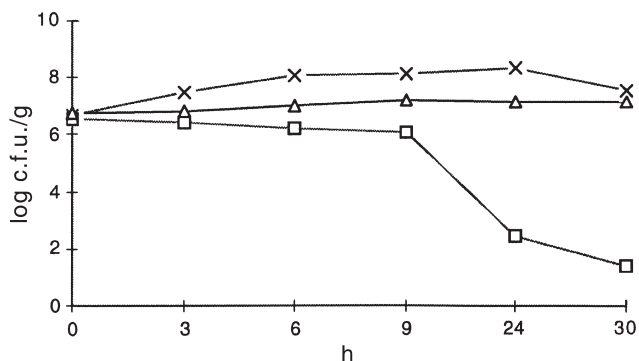


Figure 6. Inhibition of *Salmonella typhimurium* in weaning foods by *Bifidobacteria* (Bifi 19). (+) Bifi-19; (□) Bifi-19 + *Salmonella*; (△) *Salmonella* control.

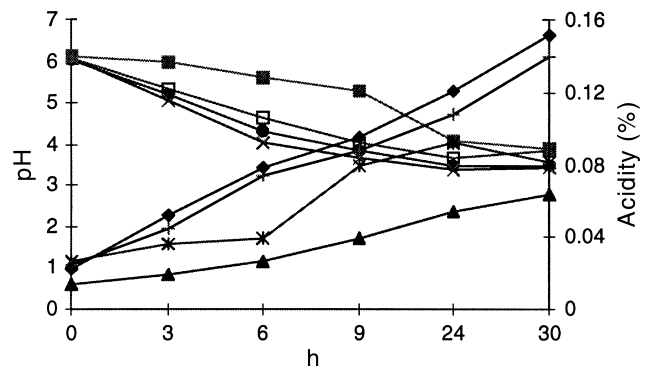


Figure 8. pH and TA in weaning foods by *Bifidobacteria* and *Salmonella typhimurium*. (□) pH Bifi-11; (●) pH Bifi-19; (×) pH Bifi-20; (■) pH *Salmonella*; (★) TA Bifi-11; (|) TA Bifi-19; (◆) TA Bifi-20; (▲) TA *Salmonella*.

Table 1. Antagonistic activity of *Escherichia coli* 0157 and *Salmonella typhimurium* S-285 by various *Bifidobacteria* strains

Strains	<i>E. coli</i> /mm	<i>S. typhimurium</i> /mm
Bifi-1	ND	ND
Bifi-2	15.0	13.0
Bifi-3	17.0	15.0
Bifi-4	17.0	15.5
Bifi-5	15.0	16.0
Bifi-6	17.0	16.0
Bifi-7	5.0	4.0
Bifi-8	14.0	13.0
Bifi-9	ND	ND
Bifi-10	14.0	16.0
Bifi-11	17.0	16.5
Bifi-12	17.0	16.0
Bifi-13	13.0	12.5
Bifi-14	14.0	13.0
Bifi-15	17.0	16.0
Bifi-16	14.5	15.0
Bifi-17	9.0	7.0
Bifi-18	5.0	6.0
Bifi-19	17.5	16.5
Bifi-20	19.0	18.0

Bifi, *Bifidobacteria*; ND, Not detected; Bifi-11, Bifi-19, Bifi-20 are *B. infantis* species, subspecies *infantis*.

on the toxicity of low pH value, due partly to the penetration of undissociated weak acids into the cells of the pathogens. When the internal pH changes, amino acid transferase RNA is inhibited and protein synthesis stops, resulting in the death of cells.²⁷ *Bifidobacteria* have been reported to have an antagonistic effect against *E. coli*, *Staphylococcus aureus*, *Shigella dysenteriae*, *Salmonella typhimurium*, *Proteus* spp. and *Candida albicans*.²⁸ The statistical analysis showed that different treatments for weaning foods had a significant (at a 1% level, $P < 0.01$) effect on the growth of *B. infantis* species (e.g. Bifi-11, Bifi-19 and Bifi-20 co-cultured with *E. coli* and *S. typhimurium*). This analysis also indicates that the populations of *E. coli* and *Salmonella* were reduced by the action of *Bifidobacteria*.

After 6 months of age, when breast milk is no longer sufficient for growing infants, infants require appropriate nutrient-rich weaning foods for growth. A practical approach to weaning is to use multimixes, which are mixtures of foods based on the staple, with other food added. In this study, the weaning food was prepared from various mixed ingredients such as sugar, sources of carbohydrates and energy; β -carotene rich DGLV powder, a mixture of amaranth species; spinach and carrot, sources of vitamin A; green gram, a source of protein supplying lysine. All such combinations could enrich the nutritive value of weaning foods. The weaning foods after inoculation with *Bifidobacteria*, *E. coli* and *Salmonella* were incubated at 35°C with a view to the effect of storage at an ambient temperature. In developing countries, where infant diarrhoeal diseases are very common in rural areas, weaning foods cannot be stored for long periods to meet the needs of the day due to lack of refrigeration facilities. These foods are stored at ambient temperatures for periods of up to 12 h for feeding to the child on demand. As a result, the food is quickly contaminated with pathogenic

organisms such as *E. coli* and *S. typhimurium* because the unhygienic conditions, poor sanitation systems and hot humid temperatures favour the growth of pathogens.²⁹ There is strong evidence that weaning foods are frequently contaminated with pathogens such as *E. coli*, *Campylobacter*, *Rotavirus*, *Shigella*, *Salmonella*, *Staphylococcus* and *Vibrio*.^{29–32}

It was found that during incubation of weaning foods at ambient temperatures the population of *Bifidobacteria* increased abruptly, but the mechanisms for this growth are not known.

The present study emphasized the preparation of a suitable method for longer shelf life of weaning foods supplemented with *B. infantis* species which could be used as potential probiotic feeds in order to control infant diarrhoeal diseases. From this study, it is hypothesized that the *B. infantis* species isolated from the breast-fed infant faeces had strong antagonistic activity against the human pathogens *E. coli* 0157 and *S. typhimurium* S 285, and that the inhibition of *E. coli* by *B. infantis* species was better than *S. typhimurium*.

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