

Full Length Research Paper

Survival of *Bifidobacterium pseudocatenulatum* G4 during the storage of fermented peanut milk (PM) and skim milk (SM) products

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Survivability of *Bifidobacterium pseudocatenulatum* G4 in fermented peanut milk (PM) and skim milk (SM) directly or supplemented with fructooligosaccharide (FOS) and human grade yeast extract was evaluated under refrigeration and 25°C for a period of 2 weeks. During the study period, the development of lactic acid and short chain fatty acids (acetic, propionic and butyric) were also examined. The fermented products were prepared utilizing inoculum of 0.67% and anaerobic incubation under control conditions using 1 l volume bioreactor to final pH less than 5. Initial mean viable number of strain G4 ranged between 7.12 - 8.35 cfu/ml fermented PM and 7.28 - 8.39 cfu/ml fermented SM based products. The viable strain G4 in fermented samples held at 25°C for 2 weeks decreased to a level of < 7 log cfu/ml due to the pH reductions, which were explained by increases in lactic, acetic, propionic and butyric acids. This final viability of the fermented products is below the requirement to claim probiotic effects upon their consumptions, while at refrigeration (4°C) storage, the viability maintained in fermented products was higher exceeding the mean recommended level of 7 log cfu viable probiotic per ml product generally. Therefore, fermented PM products especially supplemented with FOS containing strain G4 could maintain probiotics effects under refrigeration for a period of 2 weeks.

Key words: *Bifidobacterium*, storage, survival, organic acids, peanut milk.

INTRODUCTION

Bifidobacterium is the predominant species of human colonic and faecal microbiota. It has been extensively introduced as probiotics in the food industry and pharmaceutical applications (Guarner and Malagelada, 2003). Probiotic bacterium is defined as living microbial supplements, which modify intestinal microbiota balance to improve health beyond inherent basic nutrition (Fullar, 1989; Schrezenmeir and de Vrese as, 2001).

The positive health effects emanating from consumption of probiotics at recommended dose of 10^{10} live cell

live cell per include, improving lactose digestion, strengthening antimicrobial activity against pathogens, shortening the duration of diarrhoea, contributing positively to nutrition, stimulating the immune system and preventing and curing intestinal diseases (Marteau et al., 2002). These claimed benefits have recently stimulated researches in isolation and screening of new strains for probiotics purposes (Prasad et al., 1999; Fooks and Gibson, 2002; Frece et al., 2005).

However, examination carried out by Adikhari et al. (2003) and Temmerman et al. (2003) have revealed poor presence or complete absence of *Bifidobacterium* in commercially claimed yogurt preparations. At present, maintaining viability of *Bifidobacterium* until the products are consumed is essential to ensure the delivery of live

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microorganisms to consumers. It is recommended that probiotics food should contain at least 6 - 8 log cfu a live probiotic/ml of the product at the time of consumption (Schuler-Malyoth et al., 1968).

In this experiment, alternative medium of peanut milk was evaluated for survivability maintenance of *B. pseudocatenulatum* G4 during the storage of fermented products, compared with that on conventional skim milk (SM) based. Further subsequent organic acids profiles of fermented products under storage were also elucidated. Peanut milk (PM) could be a potential delivery means for probiotic strain G4. It is characterized as a source of high vegetable proteins, low in cholesterol, thus has attracted much attention from consumers. Because it is also extremely rich in minerals and essential fatty acids, such as linoleic and oleic, which are highly valuable in human nutrition (Diarra et al., 2005). *B. pseudocatenulatum* G4 previously has been screened for probiotic purposes (Shuhaimi et al., 2001; Shuhaimi et al., 2002). The strain found to survive the harsh gut environments (Wong et al., 2006), safe in *in vitro* application to mice (Kabeir et al., 2008a; 2008b), and able to utilize prebiotics in growth medium particular, fructooligosaccharides (FOS) (Shuhaimi et al., 2009).

MATERIALS AND METHODS

Preparation of fermentation mediums

Peanut beans were cleaned, washed with distilled water and soaked for 8 h at 45°C using a temperature controlled water bath. The beans were then hand hulled and blended with 1 l stainless steel warning commercial blender at medium speed for 3 min.

The obtained slurry was filtered through double-layered cheese-cloth to prepare the peanut milk (PM), which was used directly or supplemented with fructooligosaccharide (FOS) and human grade yeast extract (Biospringer, Maissonc-Alfort Cedex, France) to a final concentration of 10% w/v. The resultant peanut milk was autoclaved for 15 min at 121°C prior to fermentation. A 10% reconstituted skim milk (SM) was prepared using skim milk (NZMP skim milk powder, Auckland, New Zealand) and the slurry was then sterilized as was done for PM.

Starter culture and *Bifidobacterium* inoculum

B. pseudocatenulatum G4 was obtained from the stock culture collection of biotechnology and functional food laboratory (Faculty of Food Science and Technology, UPM, Malaysia). The strain was maintained at -20°C in 20% (v/v) glycerol and skim milk supplemented with yeast extract. A working culture was prepared by activation of the frozen strain. It was grown in skim milk (10%, w/v) supplemented with yeast extract (0.05%), incubated under anaerobic conditions using Anaerocult® A GasPac system (Merck, Darmstadt, Germany) for 48 h. Active cells were then transferred twice at 1% inoculum rate (v/v) in skim milk yeast extract medium followed by anaerobic incubation at 37 °C for 24 h prior to use.

Batch cultivation conditions

Fermentation was carried out in batches using a 4 vessels bioreactor with a temperature controlled water bath (Jeio Tech Desk

Top, Korea) and an electronic stirrer (Gas-Col Ltd, USA). The media used for fermentation were PM and SM. They were placed in clean vessels and autoclaved for 15 min at 121°C. The contents were then mixed to equilibrate the temperature to 37°C. Inoculations were next carried out with 0.67% (v/v) *Bifidobacterium* strain G4 starter culture. The initial pH was set approximately at 6.5 using human grade sodium bicarbonate and the fermentation was run to a final pH less than 5. Oxygen free nitrogen was used during fermentation to create the anaerobic environment. During the fermentation, the growth media were homogenized by stirring at speed of 200 rpm/min.

Shelf life study on fermented products

Fermented samples with high viable counts of strain G4 were collected for storage evaluation. The collected samples were stored at 4°C (refrigeration) and 25°C for a period of 2 weeks. During storage, at weekly interval samples were collected to determine the viable population, pH and individual organic acids profile including, lactic, acetic, propionic, and butyric acids.

Microbiological analysis

Samples were collected aseptically at initial (0 day) and weekly post incubation up to the second. Growth of *Bifidobacterium* was enumerated in colony forming units (CFU) per ml using the plate count method. De Man Rogosa Sharpe (MRS) agar containing 0.05% L-cysteine was used for enumeration.

Organic acid determination

1 ml of liquid fermented medium was centrifuged at 10000 rpm for 15 min. The supernatant was filtered through a 0.22 µm membrane filter and stored (-20°C) for analysis of organic acids and total soluble solids.

Profiles of organic acids were analyzed by high-performance liquid chromatography [(HPLC) Shimadzu LC-10AS Liquid Chromatography, Japan] with a Shimadzu SPD-10AV UV-VIS detector. An organic column, packed with 9 µm of polystyrene divinylbenzene ion exchange resin (Aminex HPX-87H, 300 mm x 7.8 mm, Bio-Rad Laboratories, USA) and maintained at 65°C was used. The UV detector was set at 220 nm and the mobile phase was 0.009 N sulphuric acid with a flow rate of 0.7 ml/min.

Statistical analysis

One-way ANOVA was performed to examine significant differences between normally distributed data. Tukey's-test was used to perform multiple comparisons between means within each specific growth medium. For abnormally distributed data randomization test was performed and the data were analyzed by Kruskal-Wallis test. Probability level of less than 0.05 was considered significant ($p < 0.05$). All data were analyzed using MINITAB statistical software (2006).

RESULTS AND DISCUSSION

Survivability of *B. pseudocatenulatum* G4 during the storage of fermented PM and SM

The viable number of strain G4 of fermented products at the beginning of the storage was in range of 7.12 - 8.39

Table 1. Survival of *B. pseudocatenulatum* G4 (log CFU/ml) during the storage of fermented peanut and skim milk with different supplements^a.

Time ^b	PM	PM + FOS	PM + FOS +YE	SM	SM + FOS	TPY
Initial	7.12 ± 0.07 ^a	8.35 ± 0.36 ^a	8.12 ± 0.43 ^a	7.28 ± 0.29 ^a	8.39 ± 0.27 ^a	8.25 ± 0.23 ^a
4 W1	7.20 ± 0.11 ^a	8.11 ± 0.25 ^a	8.02 ± 0.15 ^a	7.24 ± 0.29 ^a	8.08 ± 0.68 ^a	8.06 ± 0.43 ^a
4 W2	6.60 ± 0.30 ^a	7.77 ± 0.32 ^a	7.20 ± 0.08 ^b	7.14 ± 0.26 ^a	7.29 ± 0.13 ^a	7.63 ± 0.69 ^a
25W1	6.93 ± 0.47 ^a	6.68 ± 0.65 ^b	6.74 ± 0.96 ^b	7.05 ± 0.24 ^a	6.68 ± 0.51 ^b	7.76 ± 0.68 ^a
25W2	5.87 ± 0.55 ^b	6.15 ± 0.27 ^b	5.10 ± 0.43 ^c	5.51 ± 0.43 ^b	5.56 ± 0.31 ^b	5.60 ± 0.61 ^b

^aValues are mean ± STD of triplicate independent runs.

Different superscript letters in the same column indicate significant ($P < 0.05$) differences between means.

PM = peanut milk, FOS = fructooligosaccharide (0.67% w/v), YE = yeast extract (0.5% w/v), SM = reconstituted skim milk, TPY= Trypticase pytone yeast extract.

^b4 W1= Storage at 4°C for a week, 4 W2= Storage at 4°C for two weeks,

25 W1= Storage at 25°C for a week, 25 W2= Storage at 25°C for two weeks.

log cfu/ml fermented PM and 7.28 - 8.39 log cfu/ml fermented SM products. The strain G4 was maintained high in fermented PM and SM in the first week of refrigeration storage (Table 1).

However, the viable number decreased slightly in the second week of the storage, with significant ($P < 0.05$) reduction in PM+FOS+YE fermented product. Fortunately, the viability of the strain in fermented products was still at a level complying with the standard population requirement for a probiotic product to benefit the host, which equivalent to 6 - 8 log cfu/ml products. This results for strain G4 is consistence with previous report on *B. longum* BB536 survivability under refrigeration storage of fermented Sudanese *Medida* beverage, where the viability of the strain was not affected for a period of 2 weeks under refrigeration (Kabeir et al., 2004).

Therefore, the temperature of refrigeration is important to preserve fermented products with high viable number of *bifidobacteria* in different fermented carriers, such as, fermented skim milk, malted rice and peanut milk. Generally, refrigeration storage was recommended to maintain the survivability of *bifidobacteria* in traditional fermented beverages (McMaster et al., 2005).

However, Akalin et al. (2004) noted a significant reduction on *B. longum* BB46 in yogurt after only 1 week refrigeration. This indicates that the viability of *Bifidobacterium* in fermented products was dependent on the carrier type and pH of the fermented products during storage. The statement of vinderola et al. (2000) is in support to this point, where the pH of 4.5 and below was found to jeopardize the viability of probiotics in yogurt stored at lower temperature of 5°C.

When the fermented PM and SM products stored at 25°C for a period of 2 weeks, significant ($P < 0.05$) reductions in viable population of strain G4 was found (Table 1). It was also found that the viable populations of *Bifido-bacterium* and *Lactobacillus* tent to decline in fermented soymilk held at 25°C, due to acids accumulation and low tolerance of some probiotics to the acidic environment (Wang et al., 2002a; Rasic and Kurman, 1983).

At this high storage temperature of 25°C, bacteria will

continue activity, thus accumulation of more metabolic products could occur. In fact, the low buffer capacity was postulated as the main factor inherited to reduce viability of a microorganism in fermented food with high level of carbohydrate and low protein profile (Edwards, 2000). In general, most bifidobacteria strains are sensitive to low pH, resulting from accumulations of acids during fermentation process, thus they are likely to encounter viability reduction during storage (Gomes and Malceta, 1999).

The main metabolic products of carbohydrate fermentation by probiotics activity are organic acids substantiated by a drop in pH of the surrounding environment. This statement was approved by the study of McMaster et al. (2005), who noted a great loss in viability of *Bifidobacterium* due to acidic injury, which justified by its lower survivability in fermented milk than in control without fermentation (Ouwehand and Salminen, 1998).

pH decline and acid accumulation during the storage of PM and SM fermented with *B. pseudocatenulatum* G4. The pH of strain G4 fermented PM and SM products at the beginning of storage was in range of 4.2667 - 4.973 (Table 2). The reduction of pH was higher in fermented samples kept at 25°C compared to refrigeration at 4°C due to induced metabolic activity of the strain by heat (storage temperature of 25°C). pH reduction of PM and SM fermented products was not significantly ($P < 0.05$) different as compared to the initial level in each specific fermented product.

Calculated result from Table 2 showed that the reduction in pH of the fermented PM and SM products during storage under refrigeration for 2 weeks were 0.47, 0.17, 0.26, 0.39, 0.19 and 0.16 for fermented PM, PM+FOS, PM+FOS+yeast extract, SM, SM+FOS and TPY, respectively. While in the same fermented products, the storage temperature of 25°C caused respective pH reduction of 0.751, 0.613, 0.5433, 0.788, 0.747 and 0.2867 pH.

The results presented in Tables 3, 4, 5 and 6 show that accumulation of organic acids in strain G4 fermented products during the storage were higher in samples kept at 25°C than in samples stored at refrigeration (4°C). The

Table 2. The pH of fermented peanut and skim milk with *Bifidobacterium pseudocatenulatum* G4 during the storage^a.

Time ^b	PM	PM + FOS	PM + FOS +YE	SM	SM + FOS	TPY
Initial	4.97 ± 0.37	4.80 ± 0.27	4.64 ± 0.12	4.99 ± 0.36	4.76 ± 0.23	4.27 ± 0.12
4 W1	4.80 ± 0.61	4.71 ± 0.29	4.63 ± 0.48	4.63 ± 0.45	4.54 ± 0.42	4.16 ± 0.06
4 W2	4.50 ± 0.20	4.63 ± 0.31	4.38 ± 0.33	4.60 ± 0.19	4.57 ± 0.37	4.11 ± 0.10
25 W1	4.54 ± 0.51	4.39 ± 0.12	4.31 ± 0.32	4.52 ± 0.37	4.57 ± 0.26	4.00 ± 0.27
25 W2	4.22 ± 0.16	4.19 ± 0.14	4.37 ± 0.06	4.21 ± 0.21	4.03 ± 0.21	3.98 ± 0.26

^aValues are mean ± STD of triplicate independent runs.

There were no significant ($P < 0.05$) differences between means in column of each treatment.

PM = peanut milk, FOS = fructooligosaccharide, YE = yeast extract, SM = reconstituted skim milk, TPY= Trypticase phytone yeast extract.

^b4 W1= Storage at 4°C for a week, 4 W2= Storage at 4°C for two weeks.

25 W1= Storage at 25°C for a week, 25 W2= Storage at 25°C for two weeks.

Table 3. Lactic acid profile (mmol/ ml) during the storage of peanut and skim milk fermented with *B. pseudocatenulatum* G4.

Time ^b	PM	PM + FOS	PM + FOS +YE	SM	SM + FOS	TPY
Initial	7.21 ± 2.23 ^a	9.66 ± 1.29 ^a	7.90 ± 0.25 ^a	6.83 ± 0.56 ^a	10.77 ± 0.16 ^a	12.30 ± 3.29 ^a
4 W1	7.38 ± 1.85 ^a	10.35 ± 0.71 ^a	9.16 ± 0.52 ^a	6.79 ± 0.42 ^a	10.38 ± 0.66 ^a	12.36 ± 2.53 ^a
4 W2	7.74 ± 2.09 ^a	10.61 ± 0.93 ^a	9.27 ± 0.43 ^a	7.87 ± 0.42 ^a	10.80 ± 0.95 ^a	12.38 ± 2.43 ^a
25W1	8.85 ± 2.91 ^a	11.25 ± 1.40 ^a	9.80 ± 0.66 ^b	8.12 ± 0.33 ^b	10.70 ± 3.25 ^a	12.41 ± 1.79 ^a
25W2	9.11 ± 2.47 ^a	11.67 ± 1.49 ^a	10.34 ± 1.08 ^b	8.18 ± 0.41 ^b	11.66 ± 4.16 ^a	13.01 ± 2.31 ^a

^aValues are mean ± STD of triplicate independent runs.

Different superscript letters in the same column indicate significant ($P < 0.05$) differences between means.

PM = peanut milk, FOS = fructooligosaccharide, YE = yeast extract, SM = reconstituted skim milk, TPY= Trypticase phytone yeast extract.

^b4 W1= Storage at 4°C for a week, 4 W2= Storage at 4°C for two weeks,

25W1= Storage at 25°C for a week, 25W2= Storage at 25°C for two weeks.

Table 4. Acetic acid profile (mmol/ ml) during the storage of fermented peanut and skim milk with *B. pseudocatenulatum* G4.

Time ^b	PM	PM + FOS	PM + FOS +YE	SM	SM + FOS	TPY
Initial	6.86 ± 0.92	12.74 ± 0.41	7.27 ± 2.15	6.92 ± 1.16	13.40 ± 0.50	15.99 ± 2.44
4 W1	7.28 ± 0.71	13.36 ± 1.28	7.71 ± 1.18	7.25 ± 0.48	13.76 ± 0.95	16.21 ± 1.84
4 W2	7.65 ± 0.56	13.66 ± 1.55	8.09 ± 1.21	7.32 ± 0.59	13.62 ± 0.37	16.89 ± 1.38
25W1	8.20 ± 0.15	13.56 ± 2.95	7.47 ± 1.33	6.87 ± 0.25	14.04 ± 0.41	17.03 ± 2.77
25W2	7.77 ± 0.53	13.73 ± 1.27	8.48 ± 2.31	7.52 ± 0.19	14.64 ± 0.39	17.19 ± 1.43

^aValues are mean ± STD of triplicate independent runs.

There were no significant ($P < 0.05$) differences between means in column of each treatment.

PM = peanut milk, FOS = fructooligosaccharide, YE = yeast extract, SM = reconstituted skim milk, TPY= Trypticase phytone yeast extract.

^b4 W1= Storage at 4°C for a week, 4 W2= Storage at 4°C for two weeks.

25W1= Storage at 25°C for a week, 25W2= Storage at 25°C for two weeks.

accumulation of lactic acid during the storage was higher in fermented PM than in SM fermented products (Table 3). Moreover, TPY was the highest lactic profile after the two weeks storage of strain G4 fermented products under refrigeration (4°C). At similar storage, the lactic profile in other fermented products was higher in SM+FOS, PM+FOS, PM+FOS+yeast extract, SM, and PM in

descending order, due to FOS and yeast extract supplementations.

Furthermore, at 25°C storage temperature of the fermented products, lactic accumulation was in a similar trend of refrigeration storage except for fermented PM, where the increase was higher than in fermented SM (Table 3). However, the increase of lactic at the end of the storage

Table 5. Propionic acid profile (mmol/ ml) during the storage of fermented peanut and skim milk with *B. pseudocatenulatum* G4.

Time ^b	PM	PM + FOS	PM + FOS +YE	SM	SM + FOS	TPY
Initial	4.33 ± 0.87	6.86 ± 1.37	8.89 ± 1.47	4.34 ± 0.91	8.81 ± 1.02	36.41 ± 1.44
4 W1	5.13 ± 1.56	7.24 ± 2.10	9.03 ± 2.31	4.63 ± 1.11	8.96 ± 0.79	36.71 ± 5.16
4 W2	5.27 ± 1.60	7.60 ± 2.09	9.33 ± 2.24	4.80 ± 0.68	9.11 ± 0.61	37.26 ± 6.56
25W1	4.66 ± 1.70	6.71 ± 2.17	9.43 ± 2.32	4.68 ± 0.92	8.98 ± 1.00	37.88 ± 8.65
25W2	5.46 ± 2.37	7.54 ± 1.88	9.82 ± 2.79	4.92 ± 1.47	9.43 ± 0.98	37.72 ± 9.02

^aValues are mean ± STD of triplicate independent runs.

There were no significant ($P < 0.05$) differences between means in column of each treatment.

PM = peanut milk, FOS = fructooligosaccharide, YE = yeast extract, SM = reconstituted skim milk, TPY= Trypticase phytone yeast extract.

^b4 W1= Storage at 4°C for a week, 4 W2= Storage at 4°C for two weeks.

25 W1= Storage at 25°C for a week, 25 W2= Storage at 25°C for two weeks.

Table 6. Butyric acid profile (mmol/ ml) during the storage of fermented peanut and skim milk with *B. pseudocatenulatum* G4.

Time ^b	PM	PM +FOS	PM +FOS +YE	SM	SM +FOS	TPY
Initial	2.70 ± 0.41	1.80 ± 1.44	1.45 ± 0.98	0.48 ± 0.10	1.22 ± 0.92	1.96 ± 0.41
4 W1	3.02 ± 0.46	2.05 ± 1.33	1.57 ± 1.18	0.58 ± 0.17	1.38 ± 1.02	1.98 ± 0.41
4 W2	3.34 ± 0.48	2.24 ± 1.39	1.63 ± 1.21	0.64 ± 0.12	1.45 ± 1.02	2.05 ± 0.46
25W1	3.04 ± 0.44	2.28 ± 1.40	1.67 ± 1.23	0.65 ± 0.17	1.54 ± 1.06	2.14 ± 0.53
25W2	3.45 ± 0.51	2.41 ± 1.24	1.75 ± 1.28	0.70 ± 0.08	1.52 ± 0.91	2.22 ± 0.51

^aValues are mean ± STD of triplicate independent runs.

There were no significant ($P < 0.05$) differences between means in column of each treatment.

PM = peanut milk, FOS = fructooligosaccharide, YE = yeast extract, SM = reconstituted skim milk, TPY= Trypticase phytone yeast extract.

^b4 W1= Storage at 4°C for a week, 4 W2= Storage at 4°C for two weeks, 25

W1= Storage at 25°C for a week, 25 W2= Storage at 25°C for two weeks

was only significant ($P < 0.05$) in fermented PM+FOS+YE and SM products, as compared to its level at the beginning of the storage.

Acetic acid profile slightly increased in the first week under refrigeration of PM and SM fermented products, and then further increased in the second week (Table 4) of the storage. The value of increase during the storage was 0.79, 0.92, 0.82, 0.40, 0.22 and 0.9 mmol/ml in fermented PM, PM+FOS, PM+FOS+YE, SM, SM+FOS and TPY, respectively. At 25°C storage of similar fermented products for a period of 2 week, acetic acid increased by 0.91, 0.99, 1.11, 0.60, 1.24, and 1.20 mmol/ml of product, respectively. However, increase of acetic in fermented PM and SM fermented products at the end of the storage period did not differ significantly ($P < 0.05$) than the initial level at the beginning of the storage.

On the other hand, there was evident of slight increase in profiles of propionic and butyric during the storage of strain G4 fermented products under refrigeration and at 25°C (Tables 5 and 6). Propionic acid in strain G4 fermented products slightly increased in the first week of the storage and then the amount approximately doubled in the second week except for SM fermented sample (Table 5). The level of propionic in fermented products at the

end of the storage periods did not differ significantly ($P < 0.05$) than the initial level at the beginning of the storage. The highest butyric accumulated sample under refrigeration was PM, followed by fermented PM+FOS, SM+FOS, PM+FOS+YE, SM, and TPY in descending orders (Table 6). Nevertheless, at 25°C storage, SM was the lowest butyric content. In fact, initial butyric acid concentration is also higher in PM and the lower in SM products.

Conclusion

In conclusion, the development of a new delivery medium for probiotic *Bifidobacterium* is encouraged. That was due to both limitation and unavailability of the conventional carrier of the dairy based or high prices of probiotic dairy products in today's food market. The developed medium such as, PM based is cheap could contribute positively to deliver *B. pseudocatenulatum* G4, besides confer additional nutritional value to the diet.

Moreover, under refrigeration storage the viability of the strain G4 in fermented PM and SM products was maintained high at a favorable level ($> 7 \log \text{cfu/ml}$) for consumer's to maintain optimum health. On the other hand, the lower accumulation of propionic and butyric in fermented

products is favored, their high profiles during the storage might affect the acceptability and preference of the final fermented products. Therefore, the findings of this evaluation have elucidated the potential of using fermented PM with FOS for delivery of strain G4.

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