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# Influence of probiotic supplemented infant formula on composition of plasma lipids in atopic infants

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**Abstract**

Probiotic therapy is a new, successful approach to alleviating allergic symptoms. In this study, our aim was to investigate whether the positive results obtained with probiotic therapy would be associated with the differential absorption and utilization of dietary PUFA. 15 infants referred to a pediatric clinic on the basis of atopic eczema were weaned to *Bifidobacterium* Bb-12 or *Lactobacillus* GG supplemented infant formula, or to the same formula without probiotics (randomized, placebo-controlled, double blind study design). In plasma neutral lipids, α-linolenic acid (18:3 n-3) proportions were reduced by the probiotic supplementation. In phospholipids, *Lactobacillus* GG supplemented formula did not influence α-linolenic acid proportions, while *Bifidobacterium* Bb-12 supplemented formula increased the proportion of α-linolenic acid; from 0.13 ± 0.03 to 0.24 ± 0.03 (mean ± SEM) ($P = 0.002$). These results show that some physiological effects of probiotics may be associated with physiological interactions between probiotics and dietary PUFA. 

*Keywords:* Probiotics; Polyunsaturated fatty acids; Atopy; Plasma fatty acids

## 1. Introduction

The incidence of allergic reactions is increasing, especially in the industrialized parts of the world [1,2]. The reasons for this increase are still unknown, although heredity and various environmental and nutritional factors have been demonstrated to be relevant.

Newborn possess a dominant humoral responsiveness (Th2 type response) and this atopic state is generally converted to a cellular immunity, possibly due to early childhood infections [3]. According to this hygiene hypothesis, early infections may prepare the immature immunity of infants to cope with allergens and therefore reduce the prevalence of allergy by skewing the Th2 response towards the Th1 response [2]. In this context, the development of intestinal microflora have also been suggested to be crucial in the maturation of immunity in infants [4,5]. Consequently, the so-called probiotic therapy in the prevention and treatment of allergic disorders in infants has been suggested [5].

An association between diet and increased incidence of allergies has also been proposed (nutritional hypothesis). Especially the increased consumption of n-6 polyunsaturated fatty acids (PUFA) (e.g. linoleic acid 18:2 n-6) has been linked to this phenomenon [6,7]. The rationale of such hypothesis is that linoleic acid as a precursor of arachidonic acid (20:4 n-6) leads to higher levels of prostaglandins (e.g. $PGE_2$) which inhibit interferon-gamma production and favor a switch to immunoglobulin E production (Th2 response) [8]. As the management of allergies commonly involves special substitute formulas, the PUFA content of these may have long lasting effects on the directing of immunity. In fact, it has recently been reported that the linoleic acid to α-linolenic acid (18:3 n-3) ratio may vary considerably among different infant formulas [9]. In addition, we have demonstrated that PUFA may also influence the functioning of probiotics [10].

Although probiotics and PUFA in the management of atopy/allergy have been considered separately, we propose that these two factors may interact with each other. To investigate this hypothesis, we carried out a randomized,

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Table 1
Baseline characteristics of the study population (mean (SD)).

| Group | Subject demographics | | | | | |
|---|---|---|---|---|---|---|
| | Age of study entry | Duration of the treatment | Weight at entry | Weight gain (2 months from entry) | Amount formula consumed/day | Sex (female + male) |
| Group GG (n = 5) | 4.5 (2.0) months | 4.4 (1.7) months | 7076 (1766) g | 1453 (630) g | 76 mL/kg | 3 + 2 |
| Group Bb-12 (n = 5) | 5.7 (2.2) months | 7.3 (0.7) months | 7662 (1411) g | 1283 (828) g | 73 mL/kg | 2 + 3 |
| Group placebo (n = 5) | 5.6 (2.1) months | 5.7 (2.0) months | 7230 (1514) g | 1492 (520) g | 75 mL/kg | 3 + 2 |
| Total mean (n = 15) | 5.2 (2.0) months | 5.8 (1.9) months | 7323 (1494) g | 1409 (629) g | 75 mL/kg | 8 + 7 |

double blind, placebo-controlled formula challenge on atopic infants to see whether the absorption and utilization of the fatty acids of infant formula is affected by supplementation of infant formula with probiotic bacteria.

## 2. Methods

### 2.1. Subjects and study design

The study involved 15 infants referred to a pediatric clinic on the basis of atopic eczema; all fulfilled the Hanifin criteria for atopic eczema [11]. All the infants were exclusively breastfed before the eczema symptoms having had no exposure to any infant substitute formula. Baseline characteristics of the study population are shown in the Table 1. Informed consent was obtained from the infants' parents and the study approved by the Ethical Committee [12].

The infants were divided (randomized double-blind study design) into three groups receiving infant formula with or without probiotic bacteria; one group received an extensively hydrolyzed infant formula (PeptidiTutteli, Valio Ltd., Helsinki, Finland), another group received the same formula supplemented with *Lactobacillus* GG (ATCC53103) and the last group the same formula supplemented with *Bifidobacterium* Bb12 (supplied by Chr. Hansen A/S, Hørsholm, Denmark). The fatty acid profile of the study formula is shown in Table 2. The probiotic bacteria were selected as their safety and efficacy in treatment of infantile diarrhea and their immunomodulatory effects are well documented, and thus they are regarded as safe [13,14]. The concentrations of probiotics in the formulas for *Lactobacillus* GG and *Bifidobacterium* Bb12 were $3 \times 10^8$ and $1 \times 10^9$ colony-forming units (CFU)/g, respectively. The amount of formulas consumed by the infants and weight gain is shown in Table 1. All formulas were well tolerated by these infants.

Table 2
The fatty acid composition of extensively hydrolysed infant formula (Peptidi Tutteli, Valio Ltd., Helsinki, Finland) (Kaila *et al*., 1999)

| Fatty acids | Mean % by wt |
|---|---|
| Σ SFA | 43,78 |
| Σ MUFA | 38,30 |
| Σ n-6 PUFA | 16,10 |
| 18:2 n-6 | 16,06 |
| 20:3 n-6 | nd |
| 20:4 n-6 | nd |
| Σ n-3 PUFA | 1,67 |
| 18:3 n-3 | 1,67 |
| 20:5 n-3 | nd |
| 22:6 n-3 | nd |

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; nd: not detected.

Blood samples were collected (EDTA-tubes) at the first clinical examination before the start of the study period and at the control clinical examination (for further details, see Table 1). The samples were centrifuged to separate the plasma and blood cells, and the plasma immediately stored at −70°C for fatty acid analysis.

### 2.2. Fatty acid analysis

Lipids were extracted from 100 μl plasma with 6 ml chloroform-methanol (2:1, v/v) using the procedure described by Folch and co-workers [15]. Solvents were evaporated, and lipids resuspended to 0.5 ml chloroform and fractionated on silica Sep-Pak columns (Waters Corp., USA) by eluting with 10 ml chloroform (neutral lipids; NL) followed by 20 ml methanol (phospholipids; PL). Both fractions were evaporated to dryness and transesterified by sodium methoxide catalysis [16].

The fatty acid methyl esters (FAME) were analyzed in duplicate with a Perkin Elmer AutoSystem Gas Chromatograph (Perkin Elmer Corp., USA) equipped with programmed split/splitless injector and flame ionization detector and controlled with the Turbochrom Navigator 4 (Perkin Elmer, San Jose, CA). Silica gas chromatography column NB-351 (HNU-Nordion Ltd., Helsinki, Finland) (L = 25 m, inner diameter = 0.32 mm, film thickness = 0.2 μm) was used for analysis. The injection volume was 1 μl, and the split valve with a ratio of 1:40 was opened after 1 min. Flow rate of the carrier gas helium was 1.7 ml/min. The column temperature program was 120°C held for 2 mins, increased at a rate of 3°C/min to 230°C, and held for 20 mins. The injector temperature was programmed from 170°C to 250°C at a rate of 200°C/min. The detector temperature was 270°C.

Peaks were identified by comparison of their retention times to those of known standard mixture (68D, NuChek

Table 3
Effects of different infant formulas on fatty acid composition of serum neutral lipids in atopic infants (mean ± SEM). The change %-columns in the table represent the % FA at the study completion compared to baseline

| | Infant formula | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Lactobacillus* GG | | | *Bifidobacterium* Bb12 | | | Regular formula | | |
| | Before | After | Change % | Before | After | Change % | Before | After | Change % |
| Σ SFA | 26.27 ± 1.26 | 25.10 ± 2.22 | 96 | 26.01 ± 0.92 | 22.21 ± 1.26 | 85* | 27.19 ± 0.44 | 22.99 ± 0.79 | 85* |
| Σ MUFA | 32.12 ± 0.99 | 36.85 ± 0.51 | 115** | 29.68 ± 1.25 | 34.43 ± 0.99 | 116** | 31.82 ± 0.95 | 38.20 ± 0.70 | 120** |
| Σ n-6 | 28.45 ± 1.51 | 29.12 ± 0.60 | 102 | 31.46 ± 1.78 | 29.31 ± 1.86 | 93 | 28.90 ± 1.40 | 28.39 ± 1.30 | 98 |
| 18:2 n-6 | 25.60 ± 1.36 | 27.73 ± 0.58 | 108 | 29.07 ± 1.88 | 27.76 ± 1.74 | 95 | 26.54 ± 1.36 | 27.14 ± 1.25 | 102 |
| 20:4 n-6 | 2.84 ± 0.25 | 1.39 ± 0.13 | 49** | 2.39 ± 0.13 | 1.55 ± 0.13 | 65** | 2.36 ± 0.24 | 1.26 ± 0.07 | 53** |
| Σ n-3 | 1.30 ± 0.08 | 0.80 ± 0.06 | 62** | 1.23 ± 0.08 | 1.11 ± 0.17 | 90 | 0.83 ± 0.08 | 1.09 ± 0.24 | 131 |
| 18:3 n-3 | 1.04 ± 0.10 | 0.76 ± 0.05 | 73* | 0.94 ± 0.07 | 0.72 ± 0.10 | 77* | 0.76 ± 0.08 | 1.04 ± 0.23 | 137 |
| 20:5 n-3 | 0.27 ± 0.06 | 0.15 ± 0.03 | 56 | 0.29 ± 0.06 | 0.39 ± 0.15 | 134 | 0.12 ± 0.01 | 0.12 ± 0.02 | 100 |
| n-6: n-3 | 22.41 ± 1.59 | 39.19 ± 4.48 | 175* | 27.20 ± 2.99 | 36.53 ± 8.69 | 134 | 39.38 ± 5.28 | 35.05 ± 5.79 | 89 |

* Significant difference in fatty acid proportion before and after supplementation within each group; $P < 0.05$.
** Significant difference in fatty acid proportion before and after supplementation within each group; $P < 0.01$.

Prep, Elysian, USA). Quantitative results are expressed as weight percentages of individual fatty acids.

### 2.3. Statistics

The results are expressed as means (SEM: standard error of mean). The changes in plasma lipid composition associated with the different infant formulas were normally distributed. Statistical differences within the groups (before vs. after) were tested with a two-tailed paired *t* test. *P* values of less than 0.05 were considered to be significant. Statistical differences between groups were assessed in two phases: firstly ANOVA was performed to see whether anything appears different between groups, and if so, a multiple-comparison procedure, i.e. Bonferroni *t*-test was used to isolate the parameters producing the different results. *P* values of less than 0.0167 have been considered to be significant. Statistical analyses was performed using the StatView 4.57 (Abacus Concepts Inc. Berkeley, CA) statistical software package.

## 3. Results

### 3.1. Inter-group differences

There were no significant differences in baseline neutral lipid proportions between the three dietary groups (Table 3) except that both probiotic supplemented groups had higher eicosapentaenoic acid (20:5 n-3) baseline proportions compared to regular formula ($p < 0.012$ for both). The proportion of eicosapentaenoic acid was retained higher in the *Bifidobacterium* Bb-12 supplemented formula group ($p < 0.013$) over the study period, while lowered back to baseline proportion in the *Lactobacillus* GG supplemented formula group. After the supplementation period, the proportion of monounsaturated fatty acids (MUFA) was also lower in the *Bifidobacterium* Bb-12 supplemented formula group compared to group consuming regular formula ($p = 0.002$).

In phospholipids (Table 4) there was considerable amount of differences in baseline proportions between the study groups. At baseline (before), the proportions of MUFA ($p < 0.004$) and linoleic acid (18:2 n-6; $p < 0.014$) were lower in both probiotic supplemented groups compared to regular formula group, and remained lower over the study period ($p < 0.002$). At the baseline, *Lactobacillus* GG supplemented formula group possessed higher $\alpha$-linolenic acid (18:3 n-3) and docosahexaenoic acid (22:6 n-6) proportions compared to two other groups ($p < 0.008$), but these differences diminished during the study period. Moreover, the baseline proportions of arachidonic acid (20:4 n-6) and eicosapentaenoic acid (20:5 n-3) were higher in the *Lactobacillus* GG supplemented formula group compared to *Bifidobacterium* Bb-12 supplemented formula group and regular formula group, respectively ($p = 0.007$ and $p = 0.015$). After the study period, eicosapentaenoic acid (20:5 n-3) proportion in the *Bifidobacterium* Bb-12 supplemented formula group was higher compared to regular formula group. ($p = 0.007$)

### 3.2. Effect of extensively hydrolyzed infant formula on plasma lipid fatty acids

The extensively hydrolyzed infant formula (regular formula) altered the neutral lipids (Table 3). The relative percentage of the total saturated fatty acids (SFA) and arachidonic acid (20:4 n-6) decreased ($p = 0.003$ and $p = 0.001$, respectively), and that of MUFA increased ($p = 0.001$) during the study period. Similarly in phospholipids (Table 4), the total MUFA increased ($p < 0.001$) and the proportion of arachidonic acid decreased ($p = 0.003$). Also, the proportion of linoleic acid (18:2 n-6) increased ($p < 0.05$),

Table 4
Effects of different infant formulas on fatty acid composition of serum phospholipids in atopic infants (mean ± SEM). The change %-columns in the table represent the % FA at the study completion compared to baseline

| | Infant formula | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Lactobacillus* GG | | | *Bifidobacterium* Bb12 | | | Regular formula | | |
| | Before | After | Change % | Before | After | Change % | Before | After | Change % |
| Σ SFA | 43.98 ± 0.56 | 46.26 ± 0.95 | 105 | 42.94 ± 0.68 | 42.35 ± 1.23 | 99 | 45.67 ± 0.53 | 45.65 ± 0.24 | 100 |
| Σ MUFA | 10.36 ± 0.27 | 14.01 ± 0.24 | 135*** | 9.61 ± 0.31 | 12.68 ± 0.59 | 132** | 11.67 ± 0.36 | 15.06 ± 0.29 | 129*** |
| Σ n-6 | 31.98 ± 0.55 | 31.01 ± 0.76 | 97 | 29.09 ± 1.02 | 30.76 ± 0.64 | 106 | 32.98 ± 0.78 | 32.42 ± 0.22 | 98 |
| 18:2 n-6 | 21.10 ± 0.69 | 24.29 ± 0.55 | 115* | 20.31 ± 0.84 | 24.03 ± 0.39 | 118* | 23.31 ± 1.08 | 25.97 ± 0.17 | 111* |
| 20:4 n-6 | 10.88 ± 0.32 | 6.72 ± 0.51 | 62*** | 8.77 ± 0.74 | 6.74 ± 0.44 | 77* | 9.67 ± 0.64 | 6.46 ± 0.18 | 67** |
| Σ n-3 | 5.59 ± 0.35 | 2.54 ± 0.29 | 45*** | 3.55 ± 0.47 | 2.23 ± 0.23 | 63 | 3.28 ± 0.35 | 1.80 ± 0.13 | 55** |
| 18:3 n-3 | 0.18 ± 0.01 | 0.19 ± 0.02 | 106 | 0.13 ± 0.03 | 0.24 ± 0.03 | 185** | 0.12 ± 0.01 | 0.21 ± 0.06 | 175 |
| 20:5 n-3 | 0.59 ± 0.09 | 0.13 ± 0.04 | 22*** | 0.46 ± 0.13 | 0.28 ± 0.07 | 61 | 0.22 ± 0.05 | 0.10 ± 0.04 | 45 |
| 22:6 n-3 | 4.81 ± 0.27 | 2.22 ± 0.25 | 46*** | 2.95 ± 0.37 | 1.71 ± 0.16 | 58* | 2.94 ± 0.30 | 1.49 ± 0.13 | 51** |
| n-/n-3 | 6.00 ± 0.50 | 13.58 ± 1.37 | 226*** | 11.42 ± 2.89 | 12.43 ± 1.86 | 109 | 12.44 ± 2.59 | 19.13 ± 1.77 | 154 |

* Significant difference in fatty acid proportion before and after supplementation within each group; $P < 0.05$.
** Significant difference in fatty acid proportion before and after supplementation within each group; $P < 0.01$.
*** Significant difference in fatty acid proportion before and after supplementation within each group; $P < 0.001$.

and the proportions of the total n-3 PUFA and docosahexaenoic acid (22:6 n-3) decreased ($p = 0.003$ and $p = 0.001$, respectively)

### 3.3. Effect of infant formula supplemented with *Lactobacillus GG* on plasma lipid fatty acids

As with the regular formula, the *Lactobacillus* GG supplemented formula increased the relative percentage of the total MUFA ($p = 0.001$) and decreased arachidonic acid ($p = 0.002$) in plasma neutral lipids (Table 3). Additionally, the total n-3 PUFA and α-linolenic acid (18:3 n-3) decreased in those infants receiving the *Lactobacillus* GG supplemented formula ($p = 0.002$ and $p < 0.05$, respectively). Consequently, the n-6 to n-3 ratio increased within the *Lactobacillus* GG group ($p = 0.02$). In phospholipids (Table 4), the total MUFA and linoleic acid increased ($p < 0.001$ and $p = 0.03$, respectively), whereas the proportions of arachidonic acid, total n-3 PUFA and docosahexaenoic acid decreased ($p < 0.001$ for each). Additionally, the *Lactobacillus* GG supplemented formula decreased the proportion of eicosapentaenoic acid (20:5 n-3) in phospholipids ($p = 0.001$). These alterations resulted in a more than 2-fold increase in n-6 to n-3 PUFA ratio ($p < 0.001$). However the decreases in arachidonic and n-3 fatty acids in serum phospholipids in infants fed the *Lactobacillus* GG formula should be interpreted with caution because of their higher baseline proportions as compared to those fed either the regular or the *Bifidobacterium* Bb-12 Formula

### 3.4. Effect of infant formula supplemented with *Bifidobacterium Bb-12* on plasma lipid fatty acids

In neutral lipids, the extensively hydrolyzed infant formula supplemented with *Bifidobacterium* Bb-12 mimicked the effects of the regular formula (Table 3); the relative percentage of the total SFA and arachidonic acid decreased ($p = 0.01$ and $p = 0.002$, respectively), and that of MUFA increased ($p = 0.02$). Also, the proportion of α-linolenic acid in neutral lipids decreased with the *Bifidobacterium* Bb-12 supplemented formula ($p = 0.03$). In phospholipids (Table 4), the *Bifidobacterium* Bb-12 supplemented formula increased the proportions of total MUFA ($p = 0.001$), linoleic acid ($p < 0.05$) and α-linolenic acid ($p = 0.002$), and decreased arachidonic acid ($p = 0.03$) and docosahexaenoic acid ($p = 0.04$).

## 4. Discussion

There are numerous studies that have evaluated the effect of PUFA supply on early infancy. Among others, the supply of PUFA during the first stages of life has profound effects on structural and functional development of the nervous system [17]. The major influence of PUFA deficiency on growth and brain development in early life have been explained by the role of PUFA as basic components of biological membranes, precursors of eicosanoids, and regulators of gene expression. In addition to the well-established role of n-6 PUFA in humans, recent studies support the pivotal nature of n-3 PUFA in early life [17].

In relation to the context of the present paper, it has also been suggested for decades that atopy is associated with disturbances in the plasma and breast milk levels of n-6 PUFA (e.g. higher levels of linoleic acid, lower levels of γ-linolenic acid and arachidonic acid) and n-3 PUFA series (reduced proportions of LC-n-3 PUFAs) [18,19]. Subsequently, the concept of a relationship between development of atopy in the infant and low n-3 PUFA levels in their diet has been suggested. In allergic infants the permeability of

the mucosal barrier is increased causing local intestinal inflammation [5,20]. Dietary factors, such as PUFA (especially n-6 PUFA, e.g. arachidonic acid 20:4 n-6) may further enhance mucosal damage by promoting intestinal inflammation [21], whereas probiotic therapy has been demonstrated to be beneficial in host protection against allergic sensitization [5,12]. Although only limited data on the effects of probiotics on dietary fatty acids has been published, there are indications that intestinal bacteria may interact with different fatty acids.

While shorter chain fatty acids, whether they occur as such in the intestine or are products of bacterial metabolism, are readily tolerated by the indigenous microflora, the situation is more complex regarding PUFA. These are not as readily utilized nor tolerated over as wide a concentration range as are the short-chain fatty acids. In low concentrations PUFA can be innocuous or even stimulatory, while in slightly higher concentrations they become highly inhibitory to bacterial growth and adhesion to intestinal surfaces [10]. In an animal model, dietary $\alpha$-linolenic acid or a mixture of n-6 and n-3 PUFA have been shown to induce the frequency of lactobacilli in the digestive tract, whereas a lower frequency of lactobacilli was found in animals fed on a linoleic acid diet [9,22]. Thus, dietary PUFA may influence the establishment of normal intestinal microflora. *Vice versa*, intestinal bacteria, and probiotics, have also been demonstrated to influence the dietary fats. Probiotics have been reputed to possess cholesterol-lowering properties in man, though the role of probiotics remains equivocal [23]. Recently, it has also been reported that intestinal bacteria could convert free linoleic acid to conjugated linoleic acid *in vitro* (CLA; 18:2, c9/t11/t9, c11) [24,25], but this metabolic activity has not been applicable *in vivo* [26, 27]. As yet, very little is known about the relationship between intestinal microflora, or probiotics, and dietary PUFA and the resulting plasma lipid composition.

Here we carried out a randomized, double blinded, placebo-controlled clinical study to demonstrate the influence of probiotics within infant formulas on the PUFA composition of plasma lipids. The fatty acid composition of the extensively hydrolyzed formula [9] consumed by the infants was reflected in their plasma lipid composition, indicating the complete shift in their diet from breast milk to the intervention formulas. The probiotic supplemented formulas resulted in similar proportional changes in plasma lipids as the regular formula, though specific effects were also observed with these test formulas. Especially, the probiotic formulas influenced the proportions of n-3 PUFA in neutral lipids; both *Bifidobacterium* Bb-12 and *Lactobacillus* GG supplemented formulas reduced the proportion of $\alpha$-linolenic acid. In the latter case, this also resulted in a lowered total n-3 PUFA proportion and subsequently increased n-6 to n-3 PUFA ratio in neutral lipids. In contrast to lowered the $\alpha$-linolenic acid proportion observed in neutral lipids, the probiotic supplementation of the infant formulas had either no effect (*Lactobacillus* GG supplemented formula) or even increased the proportion of $\alpha$-linolenic acid in phospholipids (*Bifidobacterium* Bb-12 supplemented formula). Still, the n-6 to n-3 PUFA ratio in phospholipids increased in *Lactobacillus* GG supplemented formula group due to a lowered eicosapentaenoic acid proportion. Though it seems that probiotic supplemented formulas resulted in specific changes in plasma lipids compared to regular formula, the mechanism by which probiotics could achieve the effects seen in the present study remains inconclusive. There are at least two possible mechanisms; (i) probiotics could influence the FA composition of the formulas during the storage by either utilizing or simply assimilating certain PUFAs, or (ii) probiotics could influence the mechanisms of dietary PUFA uptake to the intestinal epithelium. Studies validating the above mentioned hypothetical mechanisms are lacking.

In this pilot study we have shown that the use of probiotic supplemented infant formula resulted in altered plasma lipid PUFA composition when compared to a regular, non-supplemented formula. As the PUFA composition of mothers' breast milk was not determined here, one can speculate whether these changes are simply due to the shift in individual diets or the result of specific interactions between probiotics and PUFA. Although these findings need to be studied further, the results presented here open up an interesting perspective for the future in producing novel, probiotic functional foods.

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