Lactobacillus acidophilus INMIA 9602 Er-2 strain 317/402 probiotic regulates growth of commensal Escherichia coli in gut microbiota of familial Mediterranean fever disease subjects

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Significance and Impact of the Study: This is the first study to demonstrate the effects of Narine, containing the probiotic Lactobacillus acidophilus, on the growth of gut commensal Escherichia coli from study participants with familial Mediterranean fever disease (FMF). Verhulst’s logistic function was demonstrated to act as a possible tool for the evaluation and quantification of effects produced by the probiotic formulation in FMF participants.

Keywords
E. coli, FMF disease, growth preparatory phase, gut microbiota, probiotic, Verhulst’s logistic function.

Abstract
Previously, we reported a positive effect the probiotic formulation, Lactobacillus acidophilus INMIA 9602 Er-2 strain 317/402 (Narine strain), had on the blood characteristics of patients with familial Mediterranean fever disease (FMF). The aim of this investigation was to evaluate the effect of the Narine probiotic on growth characteristics in the predominant commensal Escherichia coli isolates from the gut microbiota in FMF-positive study participants. Bacterial growth of 192 prevalent commensal E. coli isolates found in the volunteer participants’ guts was evaluated using Verhulst’s logistic function. This study showed that the duration of the preparatory growth phase for the E. coli isolates collected from FMF-positive volunteers was significantly shorter, whereas the duration of the logarithmic growth phase was significantly longer (P < 0.03) than that of the isolates collected from healthy participants. The Narine probiotic formulation caused a significant extension (P < 0.001) of the preparatory growth phase in the commensal E. coli isolated from FMF subjects a month after the Narine probiotic administration was terminated. The data suggest that the mathematical model characterizes the growth of commensal E. coli isolates from FMF-positive participants and it can be useful in a decision-making process on the practical use of probiotics during FMF.

Introduction
Intestinal microbiota plays an important role in human health. It is also a crucial factor in certain pathological disorders, including inflammatory bowel disease (IBD) and familial Mediterranean fever (FMF) (Silva et al. 2015). Despite the high number of valuable studies, ongoing research on FMF is of tremendous importance due to the high frequency of the disease in Armenian populations (Sarkisian et al. 2008).
Our previous investigations have shown differences in the growth parameters of commensal *E. coli* isolated from the gut microbiota of breast cancer patients (Mirzoyan et al. 2006) and patients with Crohn's disease (Gasparyan et al. 2013) in comparison with healthy study participants. The mechanisms by which the host physiological condition affects the gut's commensal growth are not fully understood yet. One of the possible mechanisms was discussed in the study by WINTER et al. (2013); it was shown that the inflammatory host response selectively influences the growth of commensal *Enterobacteriaceae* by generating electron acceptors for anaerobic respiration.

Mathematical modelling has been used to quantitatively describe the growth of microbial populations under various conditions (e.g. BELKINA et al. 2011; LOGVENKOV 2011). Modelling the growth of microbial populations allows for the controllable cultivation of micro-organisms and acts as a tool to effectively monitor and predict the microbial growth in natural and artificial biological communities (ADAIR et al. 1989; GIBSON et al. 1989). Numerous mathematical models describe microbial growth (GIBSON et al. 1989; WITZES et al. 1995; FUJIKAWA and MOROZUMI 2005; PLA et al. 2015; SKANDAMIS and JEANSON 2015). A commonly used model to characterize the growth of bacteria is the logistic function of Verhulst (PELEG and Corradini 2011). The advantage of this mathematical model is the small number of parameters it uses to provide distinct biological meanings. The calculation of the critical points of growth provides a clear identification of the different growth phases in microbial populations that correspond to different physiological states of the bacterial cells.

Probiotic therapy is a valid approach for restoring integrity and functionality to the gut microbiota (e.g. GOULET 2015). *Lactobacillus acidophilus* INMIA 9602 Er-2 strain 317/402 was isolated by YERZINKYAN in 1963 from the faeces of a healthy new-born infant and named it Narine after his daughter (YERZINKYAN 1971). This strain produces a small anti-microbial peptide (bacteriocin acidocin LCHV) which has a broad spectrum of activity against human pathogens, including meticillin-resistant *Staphylococcus aureus* and *Clostridium difficile* (MKRTCHYAN et al. 2010). The strain's clinically proven positive effects have been shown in several studies. The Narine formulation was shown to have a positive effect when used for the treatment of gastrointestinal dysbacteriosis in 200 volunteers (Dekhtsunian and Ambartsumian 1990). It also assisted in re-establishing the normal vaginal microbiota in 500 patient volunteers treated for vaginitis (Arakelian 2001). Previously, we showed that the C-reactive protein (CRP) levels in the blood of FMF patients, who were reported to have high CRP levels in remission, normalized after the Narine probiotic therapy (BALAYAN et al. 2015; PEPOYAN et al. 2015). In addition, we identified the increase in operational taxonomic units of *Escherichia* isolates in the gut microbiota of these patients (PEPOYAN et al. 2015). The Narine formulation of *Lactobacillus acidophilus* INMIA 9602 Er-2 strain 317/402 is one of the main probiotics used to improve the gastrointestinal condition of Armenian FMF patients (PEPOYAN et al. 2015).

Therefore, the aim of this study was to investigate whether the consumption of the probiotic Narine would have an impact on the growth characteristics of commensal *E. coli* isolates from the gut microbiota of FMF-positive study participants using Verhulst’s function as a tool for quantification.

**Results and discussion**

**Calculation of critical points in Verhulst’s equation**

For the approximation of empirical growth curves, the logistic function of Verhulst was used in the following form to calculate the growth of gut commensal *E. coli* (GASPARYAN et al. 2013) (function 1):

\[ X = \frac{(A - C)}{\left(1 + 10^{\alpha \beta t} \right)} + C, \]

where *X* is the optical density at time *t*, *A* is the asymptote, maximal optical density, *C* is the initial value of optical density, *t* is the total cultivation time, *α* and *β* are kinetic parameters that define the shape, point of inflection and slope of the curve.

After experimental growth data were collected, the theoretical curves and critical points for every isolate were obtained using approximation, as it is shown in Fig. 1 for isolates Healthy 1-1 and FMF Participant 1-1. Parameters *α* and *β* were determined from the linear anamorphosis of function 1. The dependence of the parameters on time *t* is described by the equation of linear regression, where *R*² is a coefficient of determination.

The first derivative of function 1 represents the absolute growth rate of the microbial population *dx/dt*.

The function has one extremum at point *T_e*, which corresponds to the maximum value of the growth rate:

\[ T_e = -\frac{\alpha}{\beta}. \]

Specific growth rate of population *μ* (h⁻¹) was calculated using the formula:

\[ \mu = \frac{dx}{dt} \cdot x^{-1}. \]

Point with coordinates (*T_e*, *x_e*) on a logistic curve corresponds to the inflection point which marks the end of
the logarithmic growth phase of the microbial population and the transition from the acceleration into the deceleration of growth ($T_e$ is the acceleration of growth is equal to zero).

The second derivative of function 1 represents the acceleration of growth. The dependence of the growth acceleration from the initial cultivation time of $E. coli$ strain has two extremes: maximum at $T_1$ and minimum at $T_2$. To find the coordinates of maximum and minimum, the third derivative of function 1 was calculated, and the values of $T_1$ and $T_2$ were obtained when the third derivative was zero.

Growth preparatory phase includes growth lag phase where growth is absent and accelerated growth phase where growth rate reaches the maximum value (until growth exponential phase). On the growth curve points, $T_1$ ($t_1, x_1$) and $T_2$ ($t_2, x_2$) are the margins of preparatory phase and the phase of maximal deceleration (negative acceleration) of the population growth, respectively. The calculation of critical points on a growth curve allows for the identification of different phases of growth in the microbial population that correspond to different physiological states in bacterial cells.

Growth characteristics of predominant $E. coli$ isolates

Table 1 shows the calculated mean values and standard errors of growth parameters of predominant $E. coli$ isolated from the FMF-positive participants. As can be seen from Table 1 and Fig. 2, the duration of the preparatory phase was on average 1.36 h for $E. coli$ isolated from the FMF participants before the probiotic Narine or placebo were administered, this is significantly shorter ($P < 0.001$) than that for strains of the healthy study participants.

The duration of the logarithmic growth phase ($T_1 - T_e$) for the $E. coli$ strains isolated from FMF-positive study participants was on average 0.68 h. The logarithmic growth phase of $E. coli$ isolated from healthy volunteers was on average 0.55 h ($P < 0.03$).

Verhulst’s logistic function was used to further characterize the growth of gut commensal $E. coli$ from the FMF-positive study participants after the intake of the Narine probiotic. These findings coincide with our previous results that used participants with the inflammatory condition, Crohn’s disease (CD) (Gasparian et al. 2013). The study described the changes in growth characteristics of commensal $E. coli$ which are similar to data in the current study.

Growth characteristics of predominant commensal $E. coli$ isolates from the gut microbiota of FMF-positive participants after probiotic therapy

The results of the investigations are presented in Table 2. There was a statistically significant increase in the duration of the preparatory phase for the predominant $E. coli$ from group ‘c’ FMF-positive participants (after Narine consumption) in comparison with those of group ‘a’ (before Narine administration): $1.79 \pm 0.055$ vs $1.29 \pm 0.11$; $P < 0.05$). No statistically confirmed differences were observed between the Placebo ‘c’ and ‘a’ groups or the Placebo ‘c’ and ‘b’ groups (where ‘b’ is 1–2 days after discontinuation of probiotics or placebo): $1.47 \pm 0.1$ vs $1.44 \pm 0.09$ and $1.57 \pm 0.074$ vs $1.47 \pm 0.1$ (Table 2).

It is known that CRP is one of the markers used to detect systemic inflammation. Previously, we reported a positive effect the Narine probiotic formulation had on FMF patients with high levels of CRP during remission (Balayan et al. 2015; Pepoian et al. 2015). It is commonly understood that gut commensals are important for
maintaining homeostasis and are essential for immunological processes treatment (Hakansson and Molin 2011).

The present study shows the effect of Narine on commensal *E. coli*, particularly the duration of the preparatory phase for *E. coli* isolates from FMF participants, was significantly lower than that for strains of the healthy study participants. The administration of the Narine probiotic formulation resulted in a significant extension of the preparatory growth phase of commensal *E. coli* isolated from FMF subjects.

Distortion of gut microbiota has been suggested a key factor in some chronic inflammatory diseases. Sartor and Mazmanian (2012) have recently reported a possible role of commensal microbiota in the pathogenesis of IBD. Growth of gut commensals might alter during the development of pathological processes (Winter et al. 2013).

The alteration of growth rate in the presence of other species illustrates that *E. coli* competes with other species (Tenaillon et al. 2010).

On the other hand, there is a complex relationship between the intestinal immune system and the gut microbiota where resident bacteria play a role in initiating and regulating both innate and adaptive immune responses. Changes in commensal bacteria could change the signals from bacteria that pass on immune cells through molecules expressed on the epithelial cell surface. Our previous studies indicated that the *E. coli* strain isolated from the gut microbiota of breast cancer patients had a significantly lower specific growth rate and reached significantly lower total biomass under aerobic and anaerobic growth conditions as compared to *E. coli* strains isolated from the gut of a healthy individual (Mirzoyan et al. 2006). We also showed that the maximal specific growth rate of commensal predominant *E. coli* isolated from patients with CD was slower than the growth of *E. coli* isolated from healthy individuals (Gasparyan et al. 2013).

Patients with FMF have used their regular anti-inflammatory medication (colchicines, 1–2 g daily). In our previous study, we demonstrated that in vitro addition of metronidazole (the antibiotic prescribed in CD therapy), which is also known to suppress cellular immunity (Miller 1980), to the culture medium did not affect the

### Table 1

Growth parameters of commensal *Escherichia coli* isolates from the gut microbiota of familial Mediterranean fever positive study participants; average ± standard error

<table>
<thead>
<tr>
<th>Isolates</th>
<th>μ_{max} (1/h)</th>
<th>Preparatory phase (h)</th>
<th>Logarithmic phase (h)</th>
<th>Max biomass (OD*)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> – healthy</td>
<td>0.71 ± 0.21</td>
<td>2.01 ± 0.11</td>
<td>0.55 ± 0.02</td>
<td>0.476 ± 0.06</td>
</tr>
<tr>
<td><em>E. coli</em> – FMF participants before probiotic Narine and placebo</td>
<td>0.84 ± 0.05</td>
<td>1.36 ± 0.08</td>
<td>0.68 ± 0.03</td>
<td>0.46 ± 0.05</td>
</tr>
</tbody>
</table>

*Optical density.
†Number of subjects.
‡Number of isolates.
P value of Student’s t-test, differences between the parameters of healthy people and FMF-positive participants. The probability P ≤ 0.05 was considered statistically significant.

![Figure 2](image-url)  

**Figure 2** Growth of gut predominant commensal *Escherichia coli* from familial Mediterranean fever (a) (prior to Narine administration) and healthy (b) volunteers. Experimental data, (♦); specific growth rate (h^{-1}), (*).
Table 2 Description of growth preparatory and logarithmic phases of commensal Escherichia coli isolates after the Narine probiotic therapy; average ± standard error

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>Healthy</th>
<th>Narine</th>
<th>Narine</th>
<th>Narine</th>
<th>Placebo</th>
<th>Placebo</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N*= 5</td>
<td>N*= 5</td>
<td>N*= 5</td>
<td>N*= 5</td>
<td>N*= 4</td>
<td>N*= 4</td>
<td>N*= 4</td>
</tr>
<tr>
<td>Nt = 30</td>
<td>n= 30</td>
<td>n= 30</td>
<td>n= 30</td>
<td>n= 30</td>
<td>n= 24</td>
<td>n= 24</td>
<td>n= 24</td>
</tr>
<tr>
<td>Preparatory</td>
<td>2.01 ± 0.11</td>
<td>1.29 ± 0.005</td>
<td>1.54 ± 0.06</td>
<td>1.79 ± 0.055</td>
<td>1.44 ± 0.09</td>
<td>1.57 ± 0.074</td>
<td>1.47 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>Pn &lt; 0.05</td>
<td>Pn &lt; 0.01</td>
<td>Pn &lt; 0.01</td>
<td>Pn &lt; 0.01</td>
<td>Pn &lt; 0.01</td>
<td>Pn &lt; 0.05</td>
<td>Pn &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>Pnsp = 0.313</td>
<td>Pnspa &lt; 0.05</td>
<td>Pnsp &lt; 0.005</td>
<td>Pnsp &lt; 0.005</td>
<td>Pnsp &lt; 0.005</td>
<td>Pnsp &lt; 0.005</td>
<td>Pnsp &lt; 0.005</td>
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<td></td>
<td>Pn &lt; 0.05</td>
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<td>Pn &lt; 0.05</td>
<td>Pn &lt; 0.05</td>
<td>Pn &lt; 0.05</td>
</tr>
<tr>
<td>Logarithmic</td>
<td>0.55 ± 0.02</td>
<td>0.66 ± 0.03</td>
<td>0.66 ± 0.03</td>
<td>0.68 ± 0.05</td>
<td>0.70 ± 0.03</td>
<td>0.66 ± 0.03</td>
<td>0.69 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Pa &lt; 0.05</td>
<td>Pa &lt; 0.05</td>
<td>Pa &lt; 0.05</td>
<td>Pa &lt; 0.05</td>
<td>Pa &lt; 0.05</td>
<td>Pa &lt; 0.05</td>
<td>Pa &lt; 0.05</td>
</tr>
</tbody>
</table>

a, before the probiotic/placebo; b, immediately after discontinuation of probiotic/placebo; c, after a month of probiotic administration was terminated; P value of Student’s t-test: Pn, differences between the parameters of healthy and FMF-positive individuals; Pnsp, differences between the parameters of FMF subjects before and after the Narine adoption; Pnsp, differences between the parameters of FMF subjects adopted Narine and Placebo; The probability P ≤ 0.05 was considered statistically significant.

*Number of subjects.
†Number of isolates.

growth parameters of E. coli isolated from healthy individuals (Pepoyan et al. 2014). On the contrary, a statistically significant difference in growth parameters was observed in E. coli isolated from CD patients, grown in the presence of metronidazole as compared to the microorganisms’ propagation in an antibiotic-free environment (Pepoyan et al. 2014), clearly indicating differences in behaviour of commensal E. coli from healthy and diseased humans.

The mathematical model utilized in this study proved to be a valid tool for biomarker analysis of commensal E. coli in FMF-positive human subjects. Also, Korshun et al. (2011) successfully used this model to investigate the influence the physiological state of E. coli cells had on the expression of the soluble protein, a recombinant analogue of glycoprotein G of Herpes simplex virus of type 2.

Thus, the administration of the commercial Narine formulation of probiotic Lactobacillus acidophilus INMIA 9602 Er-2 strain 317/402 to FMF volunteers caused significant extension of the preparatory growth phase in gut commensal E. coli as compared to the E. coli isolated from FMF patients without administration of Narine. The Verhulst’s equation utilized in this study appears to be a valid tool for biomarker analysis of commensal E. coli in FMF-positive human subjects. In addition, the use of the mathematical model provided an opportunity to calculate the primary endpoints and functional parameters of the growth of the commensals. The demonstrated effects of probiotic bacteria on the growth of E. coli can serve as one of the criteria for the intended use of probiotics in FMF patients. The probiotic bacteria can be utilized in developing therapeutic interventions aimed at restoring gastrointestinal tract immunity and integrity and modifying the intestinal microbiome to a healthy environment.

Materials and methods

Ethics

The Ethics Committee at the Ministry of Education and Science of Armenia approved these investigations prior to the start of the studies. All investigated participants provided written informed consent prior to the start of the study.

Subjects

Nine FMF (five males and four females) and five healthy volunteers (two males and three females) participated in this study. The age range of participants was 18–50 years. The FMF status of this study’s participants was confirmed through genetic analysis. Participants were not treated with antibiotics, probiotics, hormones or chemotherapeutic agents for at least a month prior to the study. The FMF-positive study participants continued their regular medication (colchicine) 1–2 g daily.

Probiotic/placebo drinks and randomization

Commercially available probiotic ‘Narine’ (Vitamax-E, Yerevan, Armenia), containing (1.5 × 10⁸) viable Lactobacillus acidophilus INMIA 9602 Er-2 strain 317/402 per capsule was administrated to FMF-positive study participants with low numbers of gut lactobacilli (about 10³–
10^4 CFU per g in faeces, this study). The gut lactobacilli were quantified during the cultural analysis using a faecal dilution method. The selective and non-selective isolation techniques were used for the culture analysis (Murray et al. 1995). Five participants were administered Narine and four served as a placebo-receiving an empty capsule. All study participants were receiving either probiotic or placebo preparation twice a day for 30 consecutive days.

Sampling and commensal E. coli isolation

Faecal materials were collected three times: the first time (point a)—before probiotic Narine or placebo was administered, the second time (point b) —immediately (1–2 days) after discontinuation of probiotics or placebo, and the third time (point c) —a month after discontinuation of probiotics or placebo. Study participants collected the faecal materials themselves in sterile plastic bags and transferred them to the laboratory on ice within 2 h of defecation. A 1 g sample of faecal material was mixed with 9 ml of phosphate-buffered saline and vortexed for 5 min) at ambient temperature, and the supernatant was serially diluted in phosphate buffer. The 10^4–10^8 dilutions were used for the isolation and enumeration of commensal E. coli isolates. Six predominant E. coli isolates from each individual and every time point were grown and investigated. The dilutions were plated on MacConkey agar for preliminary identification of Enterobacteriaceae, with further analysis using the selective media and conventional biochemical testing (Holt et al. 1994; Murray et al. 1995).

E. coli growth conditions and measurements

Luria Bertani medium (1% bacto-tryptone, 0.5% bacto-yeast extract (Difco Laboratories, Detroit, MI, USA), 1% NaCl, pH 7.5) was used to propagate E. coli cells. Bacteria were grown anaerobically as previously described (Stepanyan et al. 2007; Balayan et al. 2010). Specifically, one loop-full of fresh 24-h cultures from LB agar were inoculated in Luria Bertani broth, the optical densities were adjusted to 0.1. Bacterial population growth was examined by increasing optical density using a spectrophotometer at a 600 nm wavelength. Measurements were recorded every 30 min until the end of stationary phase.

Statistical analysis

Statistical processing of data was performed using Mann–Whitney’s test and Student’s t-test on EXCEL software, the probability P ≤ 0.05 was considered statistically significant.

Acknowledgements

This work was supported by International Science and Technology Center (ISTC) Projects A-1980 & A-2134.

Conflict of Interest

The authors have no conflict of interest to declare.

References


