

Some Peculiarities of Growth and Functional Activity of *Escherichia coli* Strain from Probiotic Formula “ASAP”

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Abstract—It has been shown that pH 7,3 and 37 °C are the optimal condition for the growth of *E. coli* “ASAP”. The cells grow well on Glucose, Lactose, D-Mannitol, D-Sorbitol, (+)-Xylose, L-(+)-Arabinose and Dulcitol. No growth has been observed on Sucrose, Inositol, Phenylalanine, and Tryptophan. The strain is sensitive to a range of antibiotics. The present study has demonstrated that *E. coli* “ASAP” inhibit the growth of *S. enterica* ATCC #700931 *in vitro*. The studies on conjugating activity has revealed no conjugant of *E. coli* “ASAP” with plasmid strains *E. coli* G35#59 and *S. enterica* ATCC #700931. On the other hand, the conjugants with low frequencies were obtained from *E. coli* “ASAP” with *E. coli* G35#61, and *E. coli* “ASAP” with randomly chosen isolate from healthy human gut microflora: *E. coli* E6. The results of present study have demonstrated improvements in gut microflora condition of patients with different diseases after the administration of “ASAP”.

Keywords—About four key words or phrases in alphabetical order, separated by commas.

I. INTRODUCTION

THE human intestinal microbiota plays a significant role in maintaining human health by preventing colonization by pathogens, degrading dietary and *in situ*-produced compounds, producing nutrients, and shaping and maintaining the normal mucosal and systemic immunity. Recently, other important functions of commensal microbiota become apparent including the influence on the lipid metabolism of the host and association with obesity as well as involvement in intestinal homeostasis, repair and angiogenesis.

Despite this seemingly robust functional redundancy built in the gastrointestinal microbiota, its integrity and function can be breached as a result of infection, antibiotic therapy, imbalanced diet, gut inflammatory disorders, cancer, surgery, and other, less obvious, factors. One of the possibilities to aid the restoration of integrity and functionality of the gut

microbiota is the use of probiotics, viable cell preparations or foods containing viable bacterial cultures or components of bacterial cells that have beneficial effects on the health of the host. Probiotics are used in curing and prevention of a raising range of diseases. The use of probiotics in animal models of inflammatory bowel disease (IBD) and in diarrhoea of premature infants, severe burn patients, and acute and chronic colitis has shown potential beneficial effects of probiotic *Escherichia coli*, *Lactobacilli*, *Bifidobacteria*, and *Saccharomyces* strains. However, the data in this area are relatively sparse and often controversial.

Probiotics have been proposed to exert their beneficial effects by maintaining a normal intestinal milieu, by stimulating the immune system, by detoxifying colonic contents, by lowering serum cholesterol levels and promoting lactose tolerance, and by producing metabolites that are essential to maintain intestinal health, reduces or eliminates ailments such as colon irritation, constipation and travelers diarrhoea, inhibition of pathogenic bacteria, synthesis of B vitamins, lowering of blood ammonia levels, cholesterol absorption, and inhibition of tumour formation [1]-[12].

The intensive researches are carried out aiming finding of new probiotic strains in different groups of microorganisms [13]. It has been shown that some *E. coli* strains exhibit the probiotic activity [14], [15]. However, the mechanisms of actions of many probiotic strains and their physiological characteristics are poorly understood yet.

Despite the long history of probiotic use, only recently the mechanisms of some probiotic effects became apparent. Among them are modulating of host cells functions and competitive exclusion of some pathogens [16]. Probiotic bacteria produce antagonistic substrates with bactericidal activity [2], [3]. Such activity exhibit some probiotic strains of *E. coli*, they produce collicins and siderofores that help them in competition with other bacteria in the gut [17], [18].

The beneficial effects of *E. coli* Nissle 1917 on the health of the host [19], [20] and supposed underlying mechanisms of action have been shown in a range of studies [21]-[23].

There are potential safety concerns with probiotics since they are live microorganisms with a potential for disease and antibiotic resistance. Some data in the literature prove the importance of risk / benefits analysis for the use of probiotics as for any other therapeutic or preventing medication [24]-[25].

In the previous study, the probiotic strain of *E. coli* has been described [15]. Despite of the conducted investigation

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concerning some growth peculiarities and characteristic of that strain [26], it seems to be important to study the optimal growth conditions, antibiotic resistance profile, and needed storage conditions of *E. coli* «ASAP». The investigation of effects of that strain on gut microflora composition of patients with different diseases are also of great interest.

II. METHODS

For all experiments *E. coli* «ASAP» probiotic strain and its lyophilized cells were used [15]. *E. coli* (ATCC#25922) was used as a control strain. The following strains - *Enterobacter aerogenes* (ATCC#35028), *Streptococcus pyogenes* (ATCC #19615) and *Salmonella enterica* (ATCC #700931), *E. coli* G35#59, G35#61 from probiotic formula Okarin [27] and two commensal strains *E. coli* E5 and E6 isolated from healthy individuals were also used [26].

Bacteria were grown in anaerobic and aerobic conditions as described earlier [26], [28]-[29]. The growth medium was LB medium (tryptone, yeast extract, NaCl, glucose) at pH 7.5, which was adjusted by using NaOH or HCl. In order to find the optimal culture temperature and pH for growth, ASAP was incubated at 5, 10, 25, 37, 42, and 46 °C, and at pH 4.35- 8.2 in LB medium.

The growth of bacteria was evaluated by estimating the spectroscopic and pH data of bacterial culture every half an hour, until it reached to the stationary state. Spectroscopic method includes measuring the optical density (OD) of the culture at 600 nm wavelength. The specific growth rate (μ) was counted in the region when increase of OD had linear dependence from time.

The lyophiliser GT-2 (Leybold-Heraeus, Vokietija) was used. After the lyophilization tightly closed products were kept 12 months at the temperature of +4°C. The number of viable cells was studied by the plate method: under the protective media; after lyophilization; and after 12 month storage at the temperature of +4°C [30].

In order to assess the role of different carbon sources in the growth of *E. coli* «ASAP» the cells have been grown in M9 medium with addition of Glucose, Lactose, D-Mannitol, D-Sorbitol, (+)-Xylose, L-(+)-Arabinose or Dulcitol [31].

The antimicrobial MICs were determined by broth microdilution according to methods described by the Clinical Laboratory Standards Institute (CLSI) [30].

For the testing of susceptibility, the next antibiotics in the following concentrations have been used: tetracycline 15µg/ml (Oxoid), doxycycline 15µg/ml (Biomerieux), amoxicillin 25µg/ml (Biomerieux), ampicillin 35µg/ml (Biomerieux), kanamycin 50µg/ml (Oxoid), gentamicin 50µg/ml (Oxoid), chloramphenicol 30µg/ml (Oxoid), streptomycin 50µg/ml (Oxoid).

To assessing the conjugative activity, *E. coli* «ASAP» has been incubated 24h at 37 °C in a growth media with 0.44 % rifampicin (Sigma, USA). Conjugative transfer of plasmids was done by filter-mating of a mix of the mid-log phase cultures of donor (*E. coli* E5 и E6, G35#59, G35#61 or *S. enterica* ATCC #700931.) and recipient (mutant *E. coli* «ASAP» strain), in a 1:1.5 ratio. The mix was incubated 18 h at 37°C and the serial dilutions were plated on selective media

with antibiotics. Conjugant cells were selected by screening of antibiotic resistance.

The number of donors and recipients were enumerated on media containing corresponding antibiotic (donor) or 135 mg/ml rifampicin (recipient). Transipients were enumerated on the medium containing both antibiotics.

Conjugation frequency in all experiments was the number of transipients divided by the number of potential recipients:

Conjugation frequency = $N/\sqrt{D} \times R$, where N - transconjugants, R - recipients, D – donors.

The healthy volunteers (N=35) and patients with FMF (N=27) and chronic colitis, gastritis, and breast cancer (N=12) with mean age of 24.4 years were enrolled in our study at the Republican Clinical Hospital and the Fanarjyans Oncology Centre in Yerevan, Armenia. None of the study participants has been treated with antibiotics, hormones, radiotherapy or any other immunosuppressive or chemotherapeutic agents for at least 2-3 weeks before the investigation.

The subjects were divided in to groups for double-blind placebo-controlled study.

The lyophilized probiotic formula *ASAP*, containing ca. 2.5×10^{10} viable cells, was administered once or twice daily, for 30 consecutive days during the investigation. The gut microbiota was analyzed 4-6 months after the discontinuation of probiotic or placebo administration.

Fecal samples were collected in sterile plastic bags and transported to laboratory on ice. Faecal material (1 g) was mixed with 9 ml of phosphate buffered saline (PBS) and vortexed for 2 min. The debris was removed by low-speed centrifugation (700xg, 5min) and the supernatant was serially diluted in PBS. The dilutions were plated on MacConkey agar (Difco, USA) for preliminary identification of *Enterobacteriaceae*, with further analysis using the selective media and conventional biochemical testing [31].

III. RESULTS AND DISCUSSIONS

Some growth peculiarities of *E. coli* strain from probiotic formula «ASAP». The study of growth parameters of intact and lyophilized cells of *E. coli* from probiotic formula «ASAP» have shown no differences in investigated parameters between intact and lyophilized cells, no differences were observed also after one-year storage of lyophilized cells (see Table I). It should be mentioned that obtained results on intact cells agree with our earlier findings [26].

It is well known that in order to provide health benefits there must be no less than 10^6 viable probiotic cells in one gram of probiotic product, and lyophilisation is a way that allows attaining that requirement. Probiotic strains vary in their ability to remain viable after lyophilizing [33].

We have shown that the probiotic strain *E. coli* «ASAP» well undergo the freeze-drying and remain viable after one year of storage.

The investigations of optimal conditions for growth of *E. coli* «ASAP» have shown that pH and T °C optimum are pH 7.3 and 37 °C, correspondingly. It has been demonstrated that 77 % of the cells kept their viability when pH was lowering up

to pH 5,35, whereas 98% of cells were growing well when temperature was 42 °C (see Fig. 1).

The roles of different carbon sources for the growth of *E. coli* "ASAP" have been assessed. It has been revealed that investigated strain grows well on Glucose, Lactose, D-Mannitol, D-Sorbitol, (+)-Xylose, L-(+)-Arabinose and Dulcitol. No growth has been observed on Sucrose, Inositol, Phenylalanine, and Tryptophan.

Revealed growth peculiarities of probiotic strain *E. coli* "ASAP" could represent the base for future studies concerning possible use of that strain as food supplement or pharmaceutical preparation.

Antibiotic resistance, conjugative activity and antagonistic potential of bacteria. The antibiotic resistance of *E. coli* "ASAP" to commonly used in practice antibiotics have been investigated (see Methods). It has been shown that investigated strain are sensitive to a range of antibiotics and corresponding MICs have been as following: to tetracycline and doxycycline – less than 1.6 µg/ml, to chloramphenicol – less than 3.4 µg/ml, to amoxicillin - less than 4.16 µg/ml, to ampicillin - less than 5.8 µg/ml, gentamicin – less than 6.25 µg/ml, streptomycin – less than 7.14 µg/ml, kanamycin – less than 8.3 µg/ml (see Table II).

TABLE I
GROWTH PECULIARITIES OF *E. COLI* "ASAP"

	Lyophilized cell, washed in		Lyophilized cells after 12 month storage	
	Intact cells	physiological saline solution before the lyophilization	Lyophilized in distilled water	Lyophilized in physiological saline solution
Duration of lag-phase (t)	1,8±0,2	1,6±0,3	1,7±0,2	1,6±0,2
Specific growth rate μ (t ⁻¹)	1,07±0,08	1,20±0,10	1,25±0,05	1,15±0,05
Maximum biomass (OD*)	1,14±0,04	1,30±0,10	1,15±0,02	1,18±0,02
Changing of pH during the growth	2,20±0,10	2,22±0,04	2,23±0,01	2,24±0,02

* - optical density

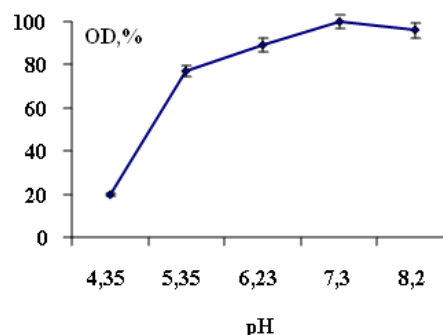
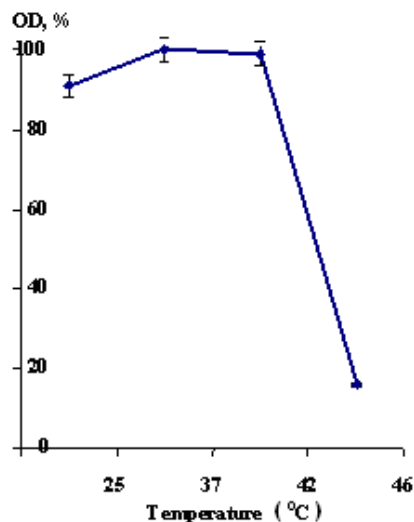


Fig. 1 Growth of *E. coli* "ASAP" under different conditions of cultivations.

TABLE II
MINIMAL INHIBITORY CONCENTRATION AND ANTIBIOTIC RESISTANCE PROFILES OF *E. COLI* "ASAP"

Strains	Antibiotics							
	Tc 15 µg/ml	Dc 15 µg/ml	Aox 25 µg/ml	Amp 35 µg/ml	Kan 50 µg/ml	Gt 50 µg/ml	Cm 30 µg/ml	Sm 50 µg/ml
<i>E. coli</i> "ASAP"	(<1,6)***	(<1,6)	(<4,16)	(<5,8)	(8,3)	(5,5)	(<3,4)	(8,3)
G35 #59	*** (30)	+ (30)	- (4,16)	+ (52,5)	- (8,3)	- (5,5)	- (3,4)	+ (350)
<i>E. coli</i> E5 [^]	- (<1,6)	- (<1,6)	- (<4,16)	- (<5,8)	- (7,14)	- (6,25)	- (<3,4)	- (7,14)
<i>E. coli</i> E6 [^]	+ (30)	+ (22,5)	- (3,57)	- (<5,8)	- (12,5)	- (6,25)	- (<3,4)	- (8,3)
G35 #61	+ (37,5)	+ (37,5)	- (<4,16)	- (5,8)	- (12,5)	- (6,25)	- (<3,4)	- (8,3)
<i>S. enterica</i> ATCC #700931	+	+	+	+	+	+	+	+
Con*	+	+	-	-	-	-	-	-

[^] - commensal *E. coli* strain isolated from healthy individual.

Tc – Tetracycline, Dc- Doxycycline, Aox- Amoxicillin, Amp- Ampicillin, Kan- Kanamycin, Gt- Gentamicin, Cm- Chloramphenicol, Sm- Streptomycin.

Con- conjugants

** (+) - normal growth, (-) - absent of growth.

***- MIC

The study of conjugating activity has revealed no conjugants of *E. coli* "ASAP" with plasmid strains *E. coli* G35#59 and *S. enterica* ATCC #700931. On the other hand, the conjugants were obtained from *E. coli* "ASAP" with *E. coli* G35#61 (three conjugants), and *E. coli* "ASAP" with E6 (one conjugant). The frequencies of conjugations were – for *E. coli* G35#61 – 0.13; for E6 – 0.05. In our previous experiments we have assessed the plasmid profiles of studied strains (results are not shown), the study has shown that probiotic strain *E. coli* "ASAP" contains no plasmid.

Antagonistic potential to pathogens are the important characteristic of probiotic strains. The present study has demonstrated that *E. coli* "ASAP" inhibits the growth of *S. enterica* ATCC #700931 *in vitro*.

The antibiotic resistance became one of the major problems in public health and veterinary [34]-[35]. It is known that gut commensals could represent the reservoir of resistant genes [36]-[37], and transient bacteria could acquire those genes via conjugation - the most important type of changing the genetic information in bacterial world, that takes place both in nature [38] and in guts of humans and animals [39]. Our results have shown that *E. coli* "ASAP" are sensible to commonly used antibiotics and do not enlarge the reservoir of antibiotic resistance in human gut.

Impact of *E. coli* "ASAP" on gut microflora composition of patients with different diseases. Clinical studies investigating commensal and probiotic bacteria are beginning to define potential therapeutic applications in the treatment and prevention of diarrhea, IBD, and other disorders [3]-[12], [40]-[43]. In addition, recently probiotics are being considered for use in control and treatment of diseases caused by pathogenic *E. coli* strains [44].

Nevertheless there are different mechanisms of action of probiotics [2], [3], [45] but it seems logical that probiotic strains of the same species as a pathogen can fight with them more efficiently, because they have similar ecological niches. In confirmation of above mentioned the recent study has demonstrated the effectiveness of probiotic *E. coli* strains in treatment of ulcerative colitis. It has been also shown that probiotic strain *E. coli* G35#59 inhibits the growth of adhesive-invasive *E. coli* strains of Crohn's disease patients *in vitro* (not published results)

Such probiotic effects as inhibition of the growth of pathogenic bacteria and improving the composition of gut microbiota has been revealed in a range of studies [46], [47].

The results of present study summarized in Fig. 2 and Fig. 3 show improvements in gut microflora condition of patients with different (see Methods) diseases after administration of "ASAP". In "ASAP" patients' group, the decrease in quantity of both antibiotic resistant *E. coli* isolates and representatives from genera *Proteus*, *Klebsiella*, *Enterobacter* and *Citrobacter* has been demonstrated. On the same time, the quantity of lactic acid bacteria has remained unchanged.

Thus, some peculiarities of growth and functional activity of *E. coli* strain from the probiotic formula "ASAP" it has been investigated.

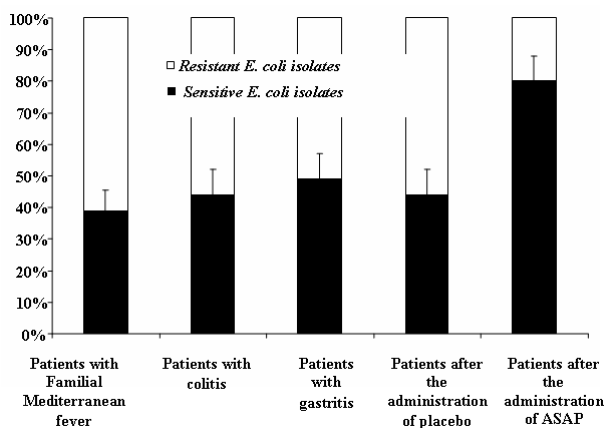


Fig. 2 Effects of administration of the probiotic on resistant isolates of *E. coli* in the guts of patients with different diseases.

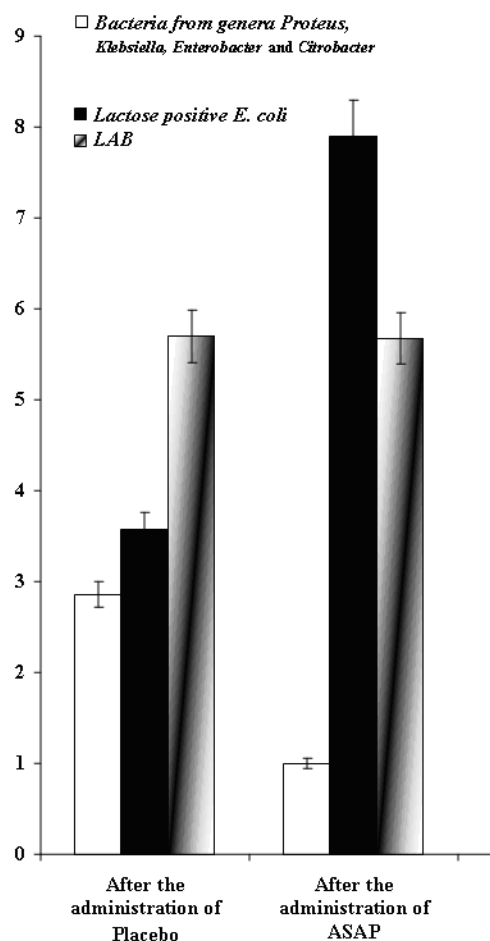


Fig. 3 Effects of probiotic therapy on guts' microflora of patients with different diseases (see Methods); the bacterial isolates in amount 10³ CFU/g belonged to one of the following genus were observed in the fecal analysis of all patients: *Proteus*, *Klebsiella*, *Enterobacter* and *Citrobacter*.

IV. CONCLUSION

Thus, the results of our investigations indicates the influence of "ASAP" on gut microflora of patients with different diseases.

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