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Protistology

SHORT COMMUNICATION

Transfer of culture of a bacterivorous protist *Nuclearia thermophila* to feeding on monoculture of *Escherichia coli*

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Summary

To quickly increase the cells number of the cultivated bacterivorous protist *Nuclearia thermophila* and minimize the diversity of bacteria in culture, for the convenience of bioinformatic processing of the sequencing results, the cultivation of *N. thermophila* was switched to feeding on monoculture of the bacteria *Escherichia coli*. Experimental selection of the medium composition showed that the PJ+WG medium with the addition of 2.5% of the LB medium ensures the propagation of *E. coli* and is suitable for rapid reproduction of many *N. thermophila* cells. A cell density of 200 cells/mm^2 was reached by the end of the first week and was about 600 cells/mm^2 by the end of the third week, with stabilization at the end of the first month at the level of ca. 700 cells/mm^2. The approximate duration of the cell cycle in the first week was about 24 hours, the rate of increase in the number of cells was 1.9 times a day. This enhancement is convenient for transcriptomic study of differential gene expression in the cell cycle. It is highly likely that in such cultivation conditions, many of the early culture cells will be at different stages of cell development.

Key words: *Nuclearia thermophila*, *Escherichia coli*, protist cultivation, cultivation media

Introduction

Genomic research of unicellular organisms usually requires obtaining and maintaining cultures of the studied organisms (Geisen et al., 2018; Faktorová et al., 2020). The cultivated culture most often has to meet two requirements. First, it must be growing fast enough so that there is always adequate material for research. Second, which is especially important for cultures used for the isolation and sequencing of nucleic acids, the culture should be sufficiently "pure", that is, contain as few organisms as possible, except for the one under study (Alkan et al., 2011; El-Metwally et al., 2013).

The bacteria that are usually present in protists' cultures play a dual role therein. On the one hand, the bacteria are food for many protists; so, their presence in cultures is often necessary (Altermatt

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et al., 2015; Amacker et al., 2022). They are usually introduced into the culture medium with the original sample and are not controlled further. On the other hand, the bacterial contamination can be a problem in nucleic acid isolation and sequencing work, especially in the cases when the diversity of bacterial species in the culture is high (Pop and Salzberg, 2008; El-Metwally et al., 2013; Cornet and Baurain, 2022). Even though the bacterial reads are separated from eukaryotic reads by bioinformatic methods using alignment to reference databases, with a large abundance and diversity of bacteria, the task of separating the bacterial reads can become more complicated, and the quality of the final data and the assembly deteriorates accordingly.

One of the options for adapting a protist culture to work with nucleic acids is the transfer of a cultivated protist to feeding on a monoculture of bacteria. The difficult step in this process is to get rid of the bacterial microflora introduced into the culture from the original sample. There are solutions using antibiotics, but the concentrations of antibiotics required to suppress the bacterial microflora often depress or even kill the cultured organism.

In this work, we transferred the cultivated *Nuclearia thermophila* Yoshida, Nakayama et Inouye, 2009 (Opisthokonta, Nucleariida), a bacterivorous protist (Yoshida et al., 2009) to the monoculture of the bacterium *Escherichia coli*. Purification from the original bacterial microflora was accomplished by selecting a medium, which stimulated the growth of the introduced bacteria and by pre-propagating the bacteria in the colonized environment, which made it possible to achieve a competitive displacement of the original bacterial species by *E. coli* (Altermatt et al., 2015).

The transition of *N. thermophila* culture to feeding on monoculture of *E. coli* had two goals: to find ways to increase rapidly the number of *N. thermophila* cells, and to minimize the diversity of bacteria in culture (ideally to a single species) for the convenience of bioinformatics processing of sequencing results.

Material and methods

Initially, *Nuclearia thermophila* cells were isolated from a sample collected in a shallow pond in the Luga region of Liningradskaya Oblast' in the Russian Federation and transferred to the PJ (Prescott's and James's) medium containing all trace elements with the addition of 0.025% wheat grass (WG) (Weizengras, Sanatur GmbH, Germany) that is organic matters necessary for protists in the amount required for bacterial growth (Prescott and James, 1955; Page, 1988). The bacteria from the natural reservoir that entered the culture multiplied therein and served as food for *N. thermophila*.

Prior to transfer to *E. coli* monoculture, *N. thermophila* was cultured on PJ+WG medium at 18 ºC and subcultured every 3 weeks by transferring 200 µl of the initial culture to a Petri dish with 15 ml of sterile new nutrient medium after which the culture was kept at 18 ºC.

For the cultivation of *E. coli*, peptone Lysogeny Broth (LB) medium, the standard liquid medium for the growth of *E. coli*, was used (Bertani, 1951; Luria et al., 1960; Sezonov et al., 2007). From the very beginning of the work, it was clear that LB was not suitable for *N. thermophila* due to its high content of animal organic matter. To grow *N. thermophila* successfully, an experimental selection of the medium composition was carried out. Specifically, *E. coli* samples were inoculated into Petri dishes containing 15 ml of PJ+WG medium or various mixtures composed of PJ+WG with the addition of LB medium in the amount of 2.5%, 5%, 7.5%, 10%, 15% and 20%. The Petri dishes with the introduced bacteria was incubated at 37 ºC for two days. After the 2 d incubation, 50 µl of the culture liquid with *N. thermophila* cells from the initial culture was transferred to the Petri dish with the grown bacteria. Three weeks later, the culture was subcultured in the same way on the same medium. The total volume of culture in each passage was 10 Petri dishes with each of the mixtures.

Microscopic observations to control the number of *N. thermophila* cells were carried out daily in the first week, then the state of the culture was checked twice a week.

Quantitative indicators (cell density, multiplication number for period and conditional multiplication number per day) were evaluated starting from the second reseeding. The assessments were quite rough and approximate, their purpose was to trace the dynamics and identify clear, indisputable differences that might have practical applicability. Therefore, the quantitative indicators of the similar cultures in all Petri dishes at the same cultivation period were averaged and the resulting values were rounded. The density of *N. thermophila* cells was assessed visually under microscopic observation by counting the average number of registered *N. thermophila* cells per 1 mm^2. The densities from 10 to 100 cells/mm^2 were rounded up to tens. Cell

densities above 100 cells/mm^2 were rounded off to the nearest number divisible by 50.

The multiplication of cell density for the certain period was estimated as:

$$\text{Multiplication number} = \text{Density (end)} / \text{Density (beginning)}$$

where Density (end) is the cell density in culture at the end of the period and Density (beginning) is the cell density at its beginning.

The multiplication in the new culture after passage for the period from inoculation to the first visual review was estimated by the formula:

$$\text{Multiplication number} = \frac{\text{Density (new)} / \text{Density (old)}}{\text{Volume of transferred liquid} / \text{Volume of culture}}$$

where Density (old) is the density of cells in the old culture from where the liquid with cells was taken, Density (new) is the density of cells in the new culture at the first review, Volume of transferred liquid is the volume of liquid with cells that was transferred from the old Petri dish to new one, and Volume of culture is the standard volume of culture liquid in one Petri dish.

This formula takes into account the decrease in cell density due to dilution of the culture and the subsequent increase due to propagation.

For each cultivation period, the average rate of cell multiplication per day was estimated. We assumed that if the number of cells in culture increases by k times per day, then in n days the number of cells will increase by $m=k^n$ times. Based on this, if for n days we noted an increase in the number of cells by m times, then we can estimate k, the coefficient of the daily increase in the number of cells, as:

$$k = \sqrt[n]{m}$$

This estimate did not take into account many factors, as well as the variability of daily cell multiplication, and was used only to compare different periods or different culture conditions.

As a result of the whole genome sequencing on the platform Illumina HiSeq 4000, aimed to obtain the *N. thermophila* genome, we also got a set of reads related to bacterial genomes. Using the Kraken2 program (Wood et al., 2019), an approximate assessment of the quantitative composition of the bacterial microflora in the culture before the introduction of the LB medium and *E. coli*, and in the culture with 2.5% LB and *E. coli* after 4 and 8 passages were made. The bacteria were identified to the genus level.

Results and discussion

When *N. thermophila* was cultivated on PJ+WG medium, individual cells were visually recorded at a density of approx. 4-5 cells/mm^2 on the 2nd or 3rd day after inoculation when observing a new culture. By the beginning of the second week, their density was about 40 cells/mm^2. This growth rate corresponds to multiplying the number of cells by 1.60 times/day, which indicates an average cell cycle length of approx. 36 h. By the end of the third week, the population density of *N. thermophila* was about 150–200 cells/mm^2 (Fig. 1, a). Thus, in the second or third week, the average rate of cell reproduction decreased to 1.11 times/day (Fig. 1, b). Further the cell density increased slightly and finally stabilized at approx. 250 cells/mm^2 after a month of cultivation. Most of the cells in culture were floating at all stages.

E. coli inoculated into pure PJ+WG media reproduced relatively slowly. After a month of cultivation, the bacteria of various morphotypes were present in culture. The rate of multiplication of *N. thermophila* cells was approximately the same as in the medium without the addition of *E. coli*, and after three weeks of cultivation, the density of their cells was ca. 200-250 cells/mm^2.

The best medium for culturing *N. thermophila* on *E. coli* as the nutrition source was the medium in which 2.5% LB was added to the PJ+WG medium. When adding 50 μL of liquid taken from a culture with a density of *N. thermophila* of 600 cells/mm^2 to a new Petri dish, the next day, individual *N. thermophila* cells (5 cells/mm^2) were visually recorded (Fig. 1, a). The concentration of 200 cells/mm2 was reached by the end of the first week (Figs 1, a; 2, a). Further, the *N. thermophila* cell density continued to increase, and by the end of the third week it was about 600 cells/mm^2 (Figs 1,a; 2, b). Subsequently, the density of *N. thermophila* cells stabilized at a level of ca. 700 cells/mm^2 after one month of cultivation. Almost all cells were at the bottom, there were very few floating ones.

Thus, on the first day, the number of *N. thermophila* cells increased approximately 2.5 times, and on the subsequent days of the first week, the rate of increase in the number of cells decreased to 1.9 times per day (Fig. 1, b). This allows calculating the approximate duration of the cell cycle as 24 h. The average rate of cells multiplication during 2 and 3 weeks was 1.07 times per day, which in total gave a threefold increase within two weeks.

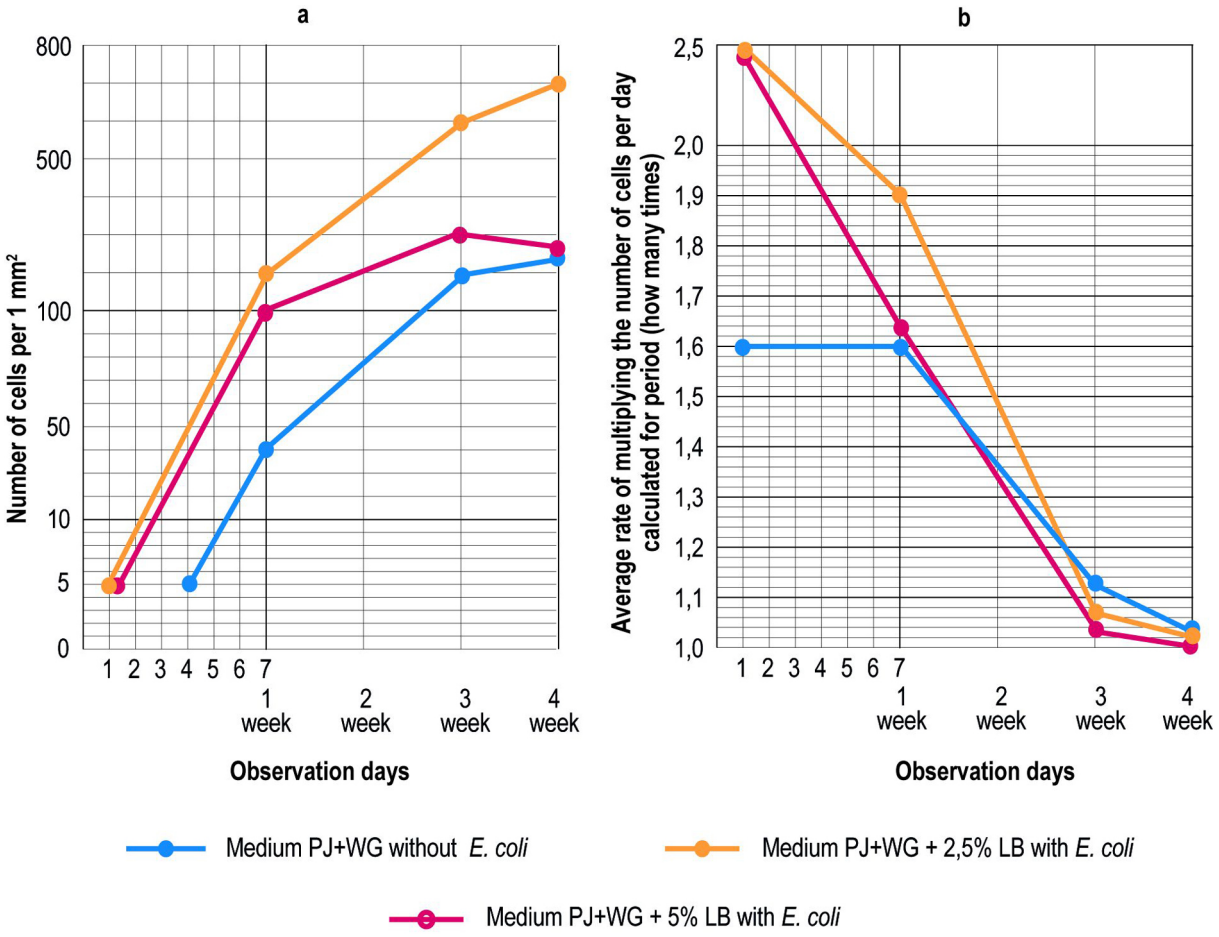

Fig. 1. Cell density (a) and the average rate of multiplication (b) for cultures of different composition during a month of cultivation.

At the same time, aggregations of the bacteria and their products in the form of strands and films were not formed in the culture. A visual decrease in the number of *N. thermophila* cells became noticeable after 1.5–2 months of cultivation.

The addition of LB in an amount of more than 2.5% resulted in excessively intensive reproduction of *E. coli*, which, in turn, led to the appearance of strands and films formed by accumulations of the bacteria and organic products. Redundant organic matter and excessive reproduction of *E. coli* inhibited *N. thermophila*. As a result, in a medium containing 15% LB or more, *N. thermophila* could not develop and its cells were not detected in the culture medium after inoculation.

At the LB concentrations of 5%, 7.5%, and 10% in the medium, *N. thermophila* first multiplied, however, the reproduction rate of *N. thermophila* was lower than in the medium with 2.5% LB. In the medium with 7.5% and 10% LB, the number of *N. thermophila* cells began to decrease from the second week and disappeared to the middle of the third week. In the medium with 5% LB, the decrease in cell density began from the third week. As a result, *N. thermophila* survived there, but by the end of the first month of cultivation, the cell density was comparable to the culture containing only PJ+WG.

Whole genome sequencing data showed that before the introduction of *E. coli* into the culture, the dominant genera of the bacteria in the culture liquid were (the percentage shows the proportion of the genus or genera in the total number of bacteria in the culture fluid) *Brevundimonas* (Alphaproteobacteria) – 15% , *Pseudomonas* (Gammaproteobacteria) – 15%, *Novosphingobium* (Alphaproteobacteria) – 5%, *Mycobacterium* (Actinomycetia) – 4%, *Mycolicibacterium* (Actinomycetia), *Azospi-rilla* (Alphaproteobacteria) – 2%, *Legionella* (Gammaproteobacteria), *Flavobacterium* (Flavobacteriia), *Streptomyces* (Actinomycetes), *Clostridium* (Clostri-

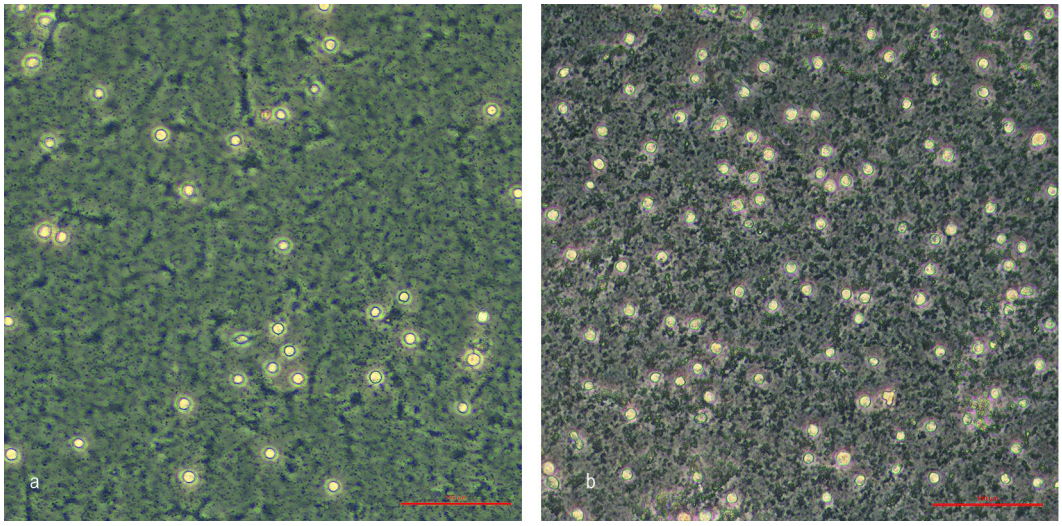

Fig. 2. Microscopic view of cultures with a cell density of about 200 cells/mm^2 (a) and about 600 cells/mm^2 (b). View at 10× magnification. Medium PJ+WG + 2.5% LB and *E. coli*.

dia) – 1%. The bacteria of other genera were also present in the culture; however, the share of each of them was less than 1%. The total share of such genera was 42% of total bacterial quantity (Fig. 3, a).

After four cultivation passages of *N. thermophila* in a medium with 2.5% LB, preliminarily enriched with *E. coli* according to the method described above, the composition of the bacterial microflora changed quantitatively and qualitatively. *E. coli* constituted 47% of total number of bacteria; the share of *Sphingomonas* (Alphaproteobacteria), which was not previously among the dominant species, was 7%; the proportion of *Brevundimonas* (Alphaproteobacteria) was 5%. The genera *Erythrobacte*r (3%), *Bradyrhizobium* (3%), *Brachybacterium* (3%), *Polaromonas* (3%), *Spirosoma* (2%), *Novosphingobium* (2%), *Paenibacillus* (1%) and *Duganella* (1%) were found among the dominant genera. The total proportion of genera, each of which is less than 1% of the total number of bacteria, was 24% (Fig. 3, b).

After 8 passages, the proportion of *E. coli* in-creased to 77% of total number of bacteria. The shares of *Sphingomonas, Brevundimonas* and *Erythrobacter* fell to 5%, 3% and 2% respectively, and the total share of genera with less than 1% was 13% (Fig. 3, c).

Our results are generally consistent with the known results of papers on the cultivation of amoeboid protists (Huws et al., 2013; Kihara et al., 2011; Kubo et al., 2013) as well as papers that noted and described the impact of organic content and diversity of nutrient organisms (Del Campo et al., 2013). As was shown in Del Campo et al. (2013), the addition of yeast organics can sharply increase the number of bacteria in the medium and the number of bacterivorous flagellates that depends on this. In laboratory cultivation, an increase in the number of cells of predatory protists was also noted when cultivating on a monoculture of other protists as a prey (Sakaguchi and Suzaki, 1999). Although these data were obtained from eukaryotic prey, the observed trend may be universal.

Therefore, the results of our work show that the previously proposed approaches are also applicable to the cultivation of the floating filose amoeba *N. termophila*. These approaches have once again yielded positive results. Thus, we have shown that they are sufficiently versatile for use in the cultivation of various bacterivorous protist, with modifications in each specific case. In addition, for the first time we have developed and tested the transfer of a protist culture to a monoculture of bacteria without the use of antibiotics, which often depress the organism under study.

Conclusions

Thus, as a result, the PJ+WG medium with the addition of 2.5% LB ensures the reproduction of *E. coli* bacteria and is suitable for rapid reproduction and obtaining many cells of bacterivorous protist *N. thermophila*. Preliminary enrichment of the medium

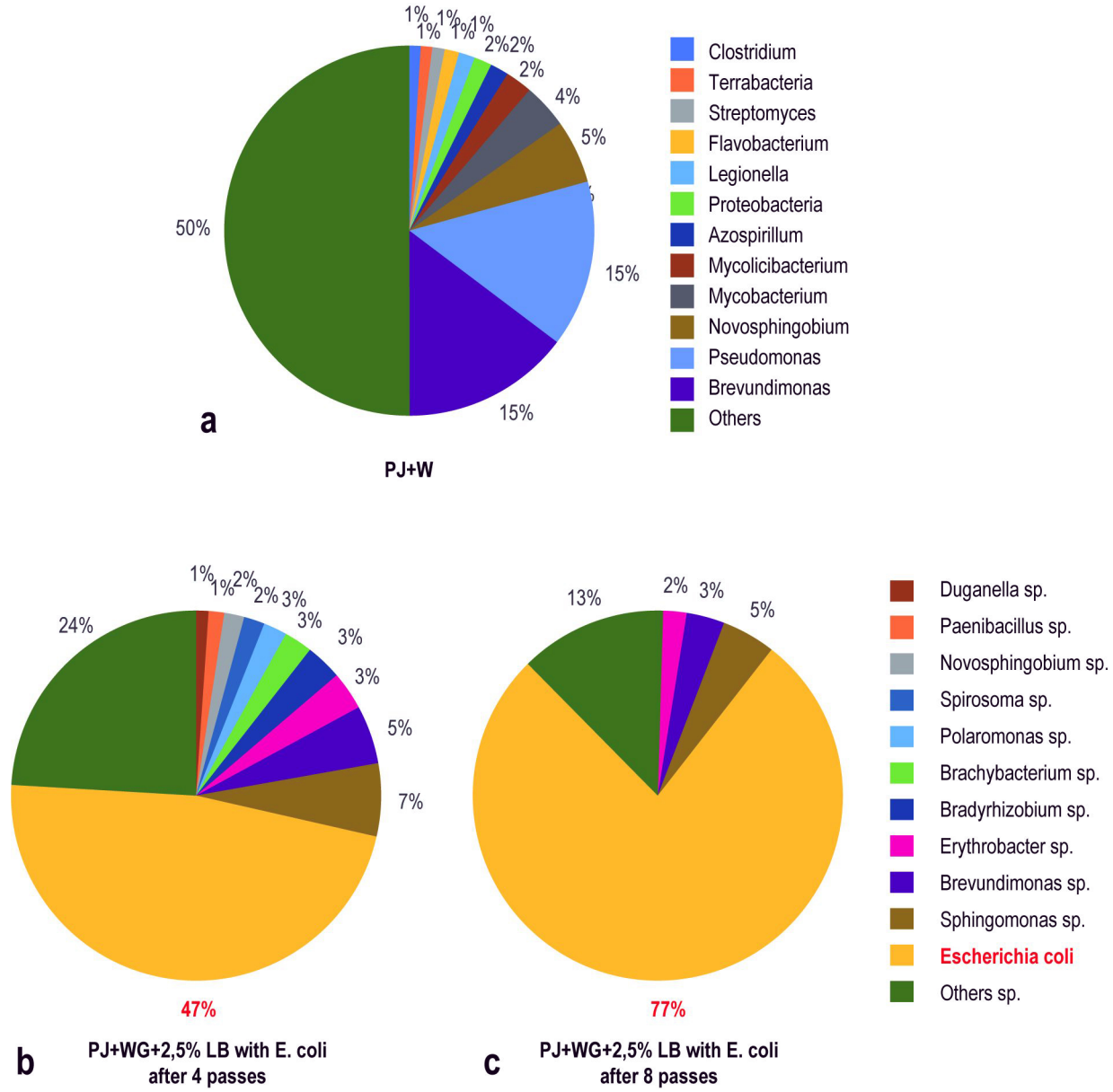

Fig. 3. Qualitative and quantitative composition of the bacterial microflora in *N. thermophila* culture in different media and passages. The percentage means the proportion of the genus or genera in the total number of bacteria in the culture fluid.

with the bacterium *E. coli* before protist inoculation provides a significant predominance of *E. coli* and a sharp decrease in the number of other bacteria after the series of passages.

This makes the system suitable for nucleic acid sequencing, as it greatly simplifies the removal of contaminating reads from the resulting sequencing outcome. Mechanic antibacterial filtration prior to nucleic acid extraction, can reduce the nucleic acid content of the bacteria other than *E. coli* to a very low level. The observed dynamics of changes in the composition of the bacterial microflora during a series of passages allows us to presume that with the continuation of the passages, the *N. thermophila* culture system could be cleared of the "natural" bacteria (coming from the original sample) and practically transferred to a monoculture of *E. coli*.

A very useful property of the resulting cultivation system is the possibility of rapid reproduction of *N. thermophila* in the first days. With a cell cycle duration of about 24 h and a rapid increase in the number of cells, it is highly likely that many of the

cells isolated from this culture in the early days will be at different stages of the cell cycle. This is convenient for transcriptomic study of differential gene expression throughout the lifespan of a single cell.

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