

# Adaptation of *Shigella flexneri* to starvation: morphology, outer membrane proteins and lipopolysaccharide changes

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**Abstract** Lack of nutrients is a common challenge for enteric pathogens, like *Shigella*, when discharged in waters and soils by infected persons. These bacteria must evolve protective mechanisms to survive nutrient changing conditions. Many studies have demonstrated that bacterial starvation can affect cell morphology and surface properties such as lipopolysaccharide content. This study focuses on the morphological and physiological alterations of *Shigella flexneri*, a human pathogen, under nutrient starvation during 30 days. Our results showed that *S. flexneri* can survive under nutrient deficiency during 4 weeks. However, starved cells revealed several morphological changes indicating decrease of the size and change of the cell shape as observed by transmission electron microscopy. Furthermore, the outer membrane proteins and lipopolysaccharide profiles of starved cells revealed the disappearance, the appearance, and changes of the protein band levels. The survival and physiological adaptation of *S. flexneri* under nutrient limitation during 30 days may increase the risk of human and animal infections.

**Keywords** *Shigella flexneri* · Starvation · Morphology · Outer membrane proteins · Lipopolysaccharides

## Introduction

Bacteria are in several ways affected by the environment, given their vital interaction with the vast surroundings. Bacteria's survival rests on a dynamic equilibrium characterized by a cycle of exchange with the environment. Bacteria are sensitive to the changes in the natural habitats, where a biotic or abiotic disturbance may be a stress factor inducing a divergence from this equilibrium (Weilharter et al. 2011; Batool et al. 2014). These disturbances stand behind the bacteria developing some mechanisms of resistance to enable an adaptation to stress and ensure its survival (Booth 2002). This adaptation can manifest itself in various ways leading to a wide range of morphological, physiological, cellular, and biochemical changes (Gustavs et al. 2009; Batool et al. 2014).

Being the first line of defense, the membrane structure is the first target of the deterioration of the physiological cells state in a stress situation (Beal et al. 2008), which explains their involvement in stress response. For the bacteria to adapt, the structure of their outer membrane undergoes numerous changes.

When they are discharged in waters and soils by infected persons, enteric pathogens are commonly challenged by the lack of nutrients. Some *Enterobacteriaceae* have developed morphological differentiation, resulting in reduced cellular forms adapted to harsh environmental conditions. Overall, during the transition from exponential growth phase and the stationary phase, the cells become spherical due to changes in the cell envelope.

*Shigella*, a Gram-negative, enteroinvasive bacterium belonging to the family of *Enterobacteriaceae*, is the causative

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agent of shigellosis or “bacillary dysentery,” a disease which is a major public health problem in both developing and industrialized countries (Phalipon and Sansonetti 2007). The annual incidence of shigellosis throughout the world is estimated at 164.7 million cases of which more than million of those infected die, with 69% of all deaths attributable to shigellosis involving children less than 5 years old (Kotloff et al. 1999; Mani et al. 2016). The disease is highly contagious due to its low infectious dose as 10 to 100 viable bacteria (Phalipon and Sansonetti 2007). *Shigella* is dispersed in the environment such as soil and wastewater. So, this pathogen is facing many potentially stressful environments during its life cycle as it is exposed to the outside environment (Nicolas et al. 2007).

In our study, we aimed to study the adaptation of *Shigella flexneri* to starvation conditions by evaluating the morphological changes, outer membrane proteins, and lipopolysaccharide profiles.

## Material and methods

### Bacterial strain and survival study

*S. flexneri* ATCC 12022 was used in this work. For the experiments, the cells were grown at 37 °C in tryptic soy broth (TSB) for 24 h, and then they were washed three times using autoclaved saline solution (0.9% NaCl) and suspended in 10 ml of autoclaved NaCl (0.9%). To prepare the laboratory microcosm, an Erlenmeyer flask containing 500 ml of NaCl (0.9%) was autoclaved and inoculated with *S. flexneri*. The microcosms were incubated under constant shaking (horizontal shaker) at 25 °C. The survival of cells was monitored weekly during 1 month by CFU determination. Triplicate determinations were made in all cases.

### Determination of morphological changes

In order to visualize the putative morphological changes in the starved strains, *Shigella* cells were examined by transmission electron microscope (TEM). For these experiments, cells were inoculated in 2 ml of nutrient broth and 0.4 ml of formaldehyde and then washed three times with 4 ml of 0.9% NaCl and centrifuged (3000×g/20 min). One drop from the final solution was deposited on the grid plus one drop of uranyl acid (Beveridge et al. 2007).

### Realization of semi-thin sections

To achieve the semi-thin sections, 15 ml of culture was prefixed with 15 ml of glutaraldehyde (GA) for 1 h at room temperature and washed three times with 0.1 M sodium cacodylate buffer at 3000×g/20 min. The pellet was

fixed with 2 ml of GA (1%) for 3 h at 4 °C. Then, the bacterial suspension was washed three times and centrifuged at 3000×g/20 min and resuspended in 0.5 ml of sodium cacodylate and 0.5 ml osmium tetroxide  $\text{OSO}_4$  (2%) for 1 h at room temperature. After washing with the cacodylate buffer and centrifugation at 3000×g/20 min, 1 ml of uranyl acetate (1%) was added and incubated for 1 h. Then, the suspension was washed first by cacodylate and second by distilled water and centrifuged at 3000×g/20 min. The residues obtained were placed at 47–50 °C with a few drops of distilled water and agar. The mixture obtained was poured on a slide, and after cooling, it was cut into small cubes which were placed in 1 ml of uranyl acetate (1%). These cubes were afterwards passed into progressive ethanol baths at increasing percentages (30, 50, 70, 90, and 100%) during 25 min for each one (Belguith et al. 2009). Then, they were placed in propylene oxide for 15 min to be delivered in propylene oxide plus epon resin at different volumes (2 vol/1 vol, 1 vol/1 vol, 1 vol/2 vol) for 15 min each one and finally in only epon during 18 h. The cubes were placed in blocks containing epon and placed at 80 °C for 2 days. The semi-thin sections of 0.5 μm, obtained using an ultramicrotome, were filed on grids and contrasted for 2 h in uranyl acetate and 5 min in the lead citrate to be examined by electron microscopy transmission.

### Outer membrane proteins (OMPs) assay

*S. flexneri* OMPs profiles were determined before and after cell incubation in the laboratory microcosms prepared according to the protocol described by Ferreiros et al. (1990). To prepare the inocula for the microcosms, *Shigella* was grown overnight in tryptic soy broth (TSB). Cell samples were taken at time 0 and after 1, 2, 3, and 4 weeks. For each sample, 50 ml was collected at the appropriate time. Briefly, cells were harvested by centrifugation at 10,000×g/4 °C during 10 min. The cell pellets were resuspended in 6 ml of acetate tampon (acetic acid 0.2 M; sodium acetate 0.2 M). The tubes were then incubated during 2 h at 45 °C under continuous shaking. The content was poured to a new tube using a syringe with 21 Ga needles promoting the membranes breaking into small vesicles. The new tubes were centrifuged at 10,000×g/4 °C during 10 min. Supernatant was collected and centrifuged at 50,000×g/4 °C during 36 min using ultracentrifuge Beckman Coulter (Rotor Type 90TI). The resulting pellet was transparent and was resuspended in 50 μl of distilled water. The concentration of OMPs in the final preparation was determined using the Bradford kit (Thermo Scientific, NanoDrop 2000). The OMPs were separated on 12% SDS gel and stained by Brilliant Blue G-Colloidal Concentrate 2025 (Sigma) (Neuhoff et al. 1988).

## Lipopolysaccharide (LPS) extraction and purification

LPS was extracted by hot phenol-water method as described by Reznia et al. (2011). In brief, bacterial suspension was centrifuged at  $10,000\times g$  during 5 min. First, the pellets were washed with phosphate buffer solution (PBS) (pH = 7.2, 0.5 M) containing  $\text{CaCl}_2$  (0.15 mM) and  $\text{MgCl}_2$  (0.5 mM) then washed by PBS only. Pellets were then resuspended in 1 ml of PBS and sonicated for 2 min on ice. In order to eliminate contaminating protein and nucleic acids, treatments with proteinase K (100  $\mu\text{g}/\text{ml}$ ), DNase (20  $\mu\text{g}/\text{ml}$ ), and RNase (40  $\mu\text{g}/\text{ml}$ ) were performed prior the extraction step.

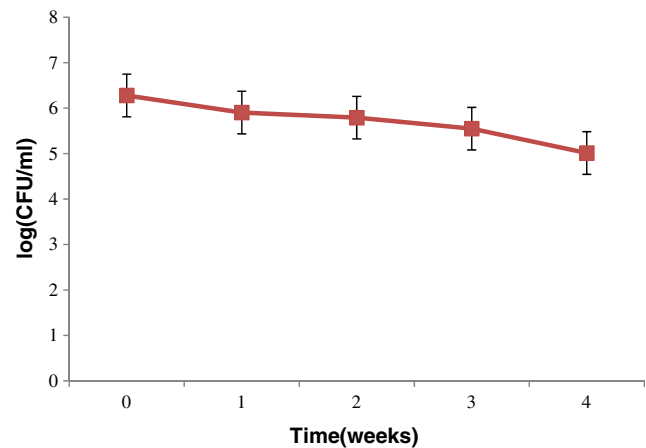
At the next step, an equal volume of hot (65–70 °C) phenol (90%) was added to the mixtures followed by vigorous shaking at 65–70 °C (15 min). Suspensions were then cooled on ice, transferred to 1.5 ml polypropylene tubes and centrifuged at  $8500\times g$  (15 min). Suspensions were transferred to 15-ml conical centrifuge tubes, and phenol phases were reextracted in 300  $\mu\text{l}$  of distilled water. Sodium acetate (0.5 M) and ethanol (95%) were added, and samples were incubated at  $-20$  °C overnight in order to precipitate LPS. Tubes were then centrifuged at  $8000\times g/4$  °C for 10 min, and pellets were resuspended in 1 ml of distilled water. Extensive dialysis against double distilled water at 4 °C was carried out until the residual phenol in the aqueous phases was totally eliminated. Final purified LPS product was lyophilized and stored at 4 °C.

The purified LPS components were separated by electrophoresis on 12% SDS gel and stained with ProteoSilver™ Silver Stain Kit (Gharahdaghi et al. 1999).

## Results and discussion

### *Shigella flexneri* survival under starvation

Relative changes in counts of *S. flexneri* during the period of starvation are shown in Fig. 1. From the first week of starvation until the fourth one, the number of viable cells of *S. flexneri* decreased slightly. Therefore, the result showed that *Shigella* was able to adapt and survive in nutritional deficiency conditions during a month of incubation in physiological water microcosms. Similar results were reported by Ellafi et al. (2011) for different strains of *Shigella* but with an important decrease of the number of viable cells since the first days of starvation. Besides, Bogosian et al. (1998) noted that the number of viable cells of some enteric bacteria decreased slowly during long period of starvation. Similar results were obtained for *Klebsiella pneumoniae* which showed a decrease in its viability (Bossolan et al. 2005). However, the viability of *Vibrio*



**Fig. 1** Survival of *Shigella flexneri* during starvation period

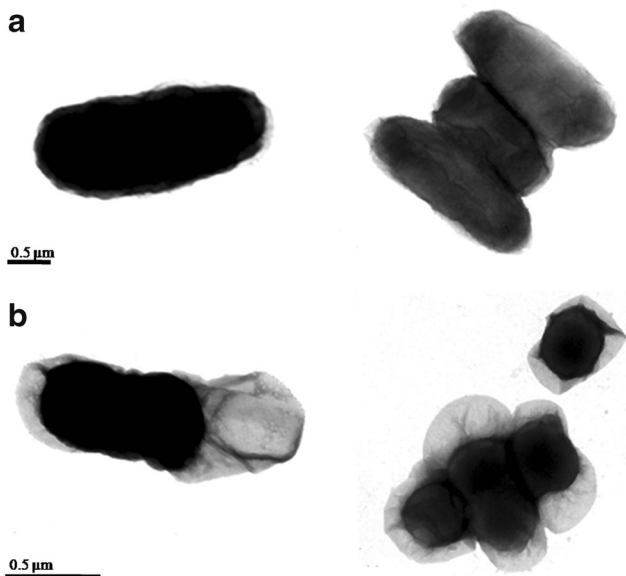
*anguillarum* decreased within 72 to 168 h of the nutritional deficiency (Nelson et al. 1997), while the number of viable cells of *Vibrio* sp. was approximately the same during 5 weeks of starvation (Amy et al. 1983).

During starvation, some bacterial species lose their cultivability but remain viable (Fakruddin et al. 2013). This phenomenon is known as viable but nonculturable (VNC): it is a survival strategy in response to adverse environmental conditions (Su et al. 2015). The VNC state has been observed in several bacterial species, and it seems that there are different molecular mechanisms trailing this phenomenon. It is characterized by low metabolic activities and some distinctive morphological changes of bacteria in the stationary phase (Li et al. 2014; Su et al. 2015; Zhao et al. 2016).

### Cell morphology of starved *Shigella flexneri*

These morphological alterations were noted in this study, when the cells of *S. flexneri* have passed from the exponential phase to starvation. We observed considerable changes in apparent structure of the cells and roughness of the outer surface. Vegetative cells of *S. flexneri* have a bacillary form up to 2–3  $\mu\text{m}$  in length with a very clear cell wall and an external membrane surrounding an expanded cytoplasm (Fig. 2a), whereas the starved cells decreased in length up to 0.5–1  $\mu\text{m}$  and transformed to a coccoid form with a condensed cytoplasm. The starved cells of *S. flexneri* turned into small spheroids via fragmentation and continuous size reduction (Fig. 2b).

The coccoid form of *Shigella* was also observed after incubation in seawater (Ellafi et al. 2011). Similarly, *K. pneumoniae* cells showed a reduction in size and a change of bacillary form to the coccoid or coccobacillus form (Bossolan et al. 2005). Further, the reduction in size was also observed in *Salmonella enterica* and *S. typhimurium* (Spector 1990; Huisman et al. 1996). In

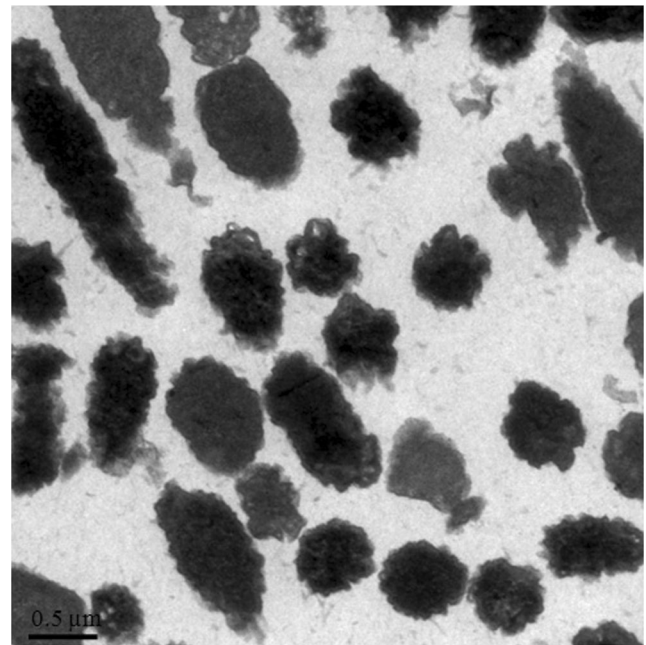


**Fig. 2** Morphology of vegetative (a) and starved cells (b) of *Shigella flexneri*

addition, after starvation, *V. cholerae* cells had serious changes of their cell morphology from curved bacilli cells to spheroid ones (Krebs and Taylor 2011). This coccoid form was described for the first time when the cells were in VNC state (Carroll et al. 2001; Chaiyanan et al. 2001, 2007; Johnston and Brown 2002). The most notable differences for the cells of *Vibrio (Listonella) anguillarum* were the reduction in the diameter by 15% and the appearance of rounded cells after 168 h of nutrient deprivation (Nelson et al. 1997). Furthermore, the conversion of the cell morphology was observed in many other pathogenic bacteria like the uropathogenic *Escherichia coli* (UPEC), which was organized into coccoid cells tucking most of epithelial cells of the urinary tract (Justice et al. 2004). Other studies of starved *E. coli* have shown that these coccoid cells can divide and exist individually or grouped (Corbin et al. 2002). This response is similar to our result for *S. flexneri*.

The observation of semi-thin sections of *S. flexneri* showed a mosaic of cells which were microcells, rounded cells, and elongated ones (Fig. 3). In addition, the result revealed that the cell envelope of the majority of stressed cells has been altered, the external and internal membranes were separated, and the cytoplasm was disorganized. Further, we observed some form of vesicles that appeared on the surface of the outer membrane. These vesicles reflect the breakdown of membrane integrity (Fig. 4).

It was demonstrated that starved cells presented numerous mutations. These mutations lead to the coexistence of many subpopulations allowing the increasing of genetic diversity (Reeve et al. 1984; Finkel and Kolter 1999). Further, the ultrastructure of starved bacterial strains



**Fig. 3** Effect of starvation on the ultrastructure of *Shigella flexneri* as demonstrated by transmission electron microscopy

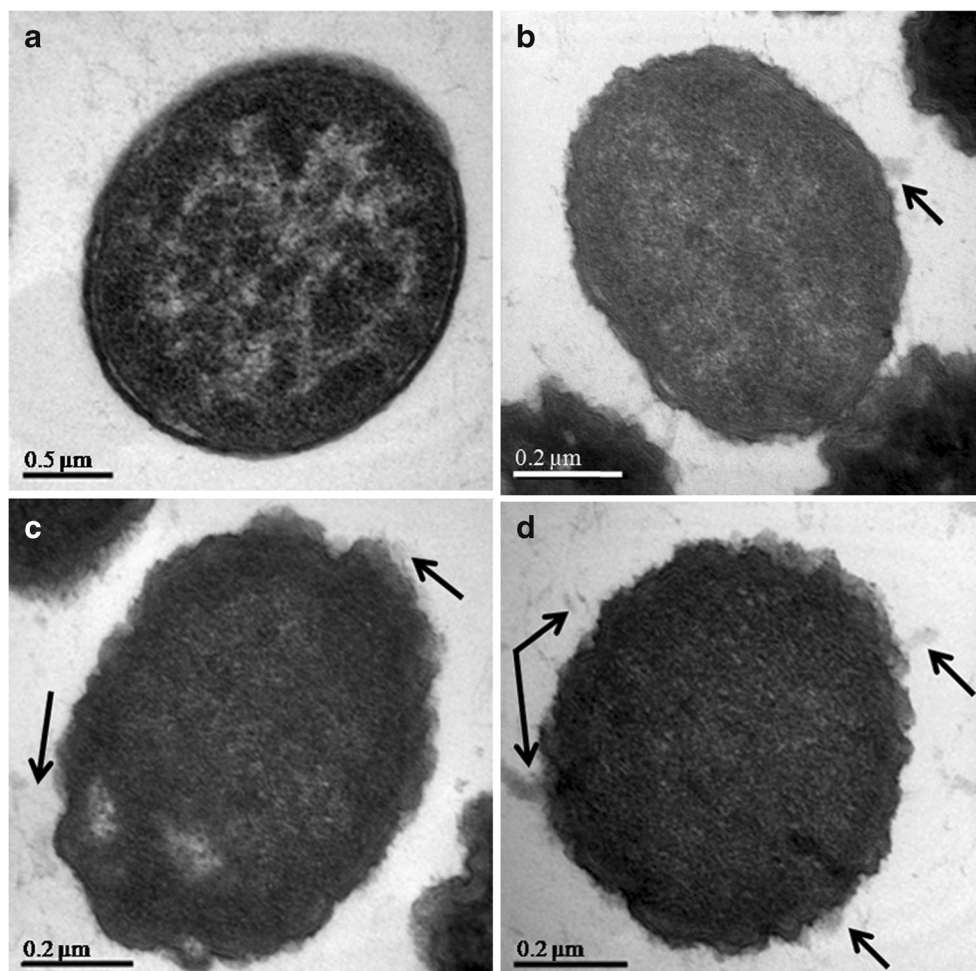
presented many morphological changes (Stretton et al. 1997; Mizunoe et al. 2000). Nelson et al. (1997) reported also that *Vibrio anguillarum* starved cells became rounded containing less coloration at the inner material and less integrity. They were elongated in curved or helical filaments. Similarly, some cells of *Vibrio* VP190 contained less inner material. They had some membrane vesicles over a portion of the outer membrane and have lost the membrane integrity. The lysis of the cell envelope and the inhomogeneous arrangement of the cytoplasm were also noted for the *Salmonella* strains after exposure to aqueous garlic extract (Belguith et al. 2009).

#### Effect of starvation on the outer membrane proteins

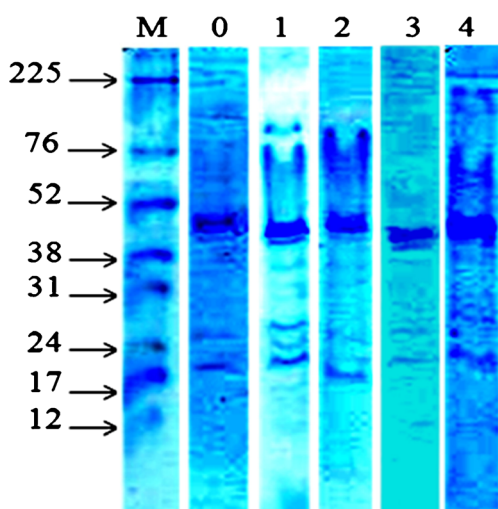
Many bacteria exhibit the expression of coordinated protective mechanisms to overcome multiple physical stresses. It is thought that the acquisition of cross protection during starvation is accomplished by the expression of starvation-stress proteins (Givskov et al. 1994a).

In our study, the starvation altered the *Shigella* OMPs. These alterations were illustrated by the disappearance, the appearance of proteins bands, and the changes of the protein band concentrations (Fig. 5). At the initial phase of starvation (T1) and at the end of the process (T4), the cells preserved some of their initial OMPs and expressed new proteins (with low level of expression) which disappeared during the T2 and T3 phases. Our results showed also that during starvation, the cells did not synthesize any protein with molecular mass up to 52 KDa and kept

**Fig. 4** Effect of starvation on the ultrastructure of *Shigella flexneri* as demonstrated by transmission electron microscopy. Control sample from exponential growth phase (a); starved cells (b, c, and d) the arrows indicate the presence of vesicles



the concentrated band corresponding to the molecular weight between 38 and 52 KDa. Similar results were obtained for starved *E. coli* (Walczak et al. 2012).



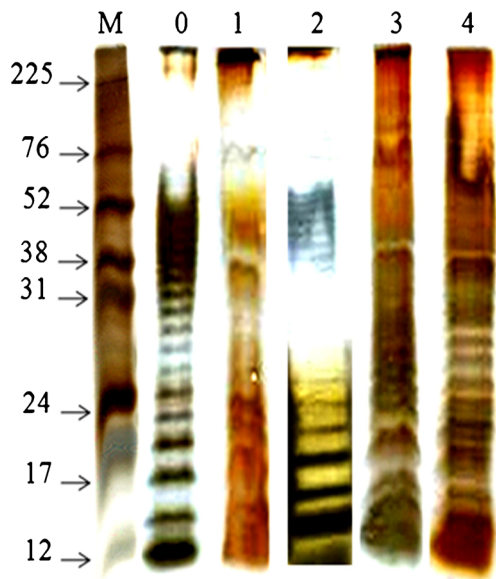
**Fig. 5** Outer membrane profiles of starved *S. flexneri*. Molecular masses are indicated by lane M (12, 24, 31, 38, 52, 76, 225 KDa). Lane 0: cells before incubation. Lanes 1, 2, 3, and 4: starved cells after 1, 2, 3, and 4 weeks

**Lipopolysaccharide profiles of starved cells**

Polyacrylamide gel is known to be affected by the number and size of repeating oligosaccharide units in long-chain LPS, such that bands in the profile represent progressively larger concatemers of the repeating oligosaccharide units (Wu et al. 2006). The starvation process showed a huge increase of bands with high expression level (Fig. 6). The LPS profiles of the *S. flexneri* showed rapid migrating bands before and during starvation with less concentrated bands in the beginning of the process. Previous studies have shown alteration of the lipid A content during starvation of marine heterotrophic bacteria (Malmcrona-Friberg et al. 1986). Also, Walczak et al. (2012) mentioned that the outer membrane LPS sugar contents did not display significant changes during starvation in *E. coli* K12.

**Conclusion**

Our results showed that *S. flexneri* is capable to survive lengthy periods under nutrient deprivation. Phenotypic and



**Fig. 6** Lipopolysaccharides of starved *S. flexneri*. Molecular masses are indicated by lane M (12, 24, 31, 38, 52, 76, 225 KDa). Lane 0: cells before incubation. Lanes 1, 2, 3, and 4: starved cells after 1, 2, 3, and 4 weeks

physiological plasticity of *S. flexneri* under starvation provides advantage to survive several stresses and results in public health risks.

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