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Detachment of adhered enteropathogenic *Escherichia coli* cells from polythene fragments immersed in aquatic microcosm using *Eucalyptus microcorys* extract (Myrtaceae)

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ABSTRACT

This study aimed to assess the effect of the concentration of an aqueous extract of *Eucalyptus microcorys* on the detachment of enteropathogenic *E. coli* cells previously adhered on polythene surface. The results showed that the extract causes a detachment of cells adhered and the importance of detachment depends on incubation duration of substrate in the plant extract solution. The higher value of detached cell abundance, after 3 hours duration of cell adhesion process (DCAP), was 8.72 (Ln (CFU/cm²)) and the percentage of detached cells reached value of 11.2% when the bacteria cells were harvested from the lag growth phase. For cells coming from exponential growth phase, after 9h of DCAP, the abundance of cells detached reached value of 8.95 (Ln (CFU/cm²)) and the higher percentage of detached cells was 68.8%. The values of 48.5% and 55.6% were noted when cells harvested from stationary and decline growth phases respectively. The cell detachment process observed could be due to the presence of some secondary metabolites such as flavonoids, triterpenoids, alkaloids, tannins which can disrupt physicochemical characteristics of bacteria surface. They seem to have a significant impact on exopolysaccharids matrix secreted by bacteria during the cell adhesion process. An overall significant difference ($P < 0.01$) was observed between the mean abundances of detached cells for each extract concentrations at different growth stages.

Keywords: *E. microcorys* extract, *E. coli* cells, detachment, adhesion, cell growth phase.

1. INTRODUCTION

Freshwater is approximately 0.6% of water resources on the planet and it is most exploited for the human feeding [1]. It can be superficial or subterranean. Surface water is exposed to various pollutants including suspended solids, microorganisms from soil, industrial and domestic waste. Due to these pollutants, lack of potable drinking water is a major factor of risk for mortality and morbidity, notably those due to diarrheal diseases [2]. Although the number of people

with access to good water quality has increased worldwide since 2000, rapid population growth impedes these improvements in many countries. Nearly one billion people still lack access to safe drinking water, half of whom live in the African and Pacific regions [2]. Bacteria known to be responsible for the microbiological water pollution included *Salmonella*, *Shigella*, *Escherichia*, *Yersinia*, *Campylobacter* and *Vibrio* species [3].

The bacterium *Escherichia coli* belongs to the group of fecal or thermotolerant coliforms [4]. Its presence in water usually indicates the deterioration of its bacteriological quality, due to the contamination by other microorganisms which could be pathogenic for human [5]. For nearly a decade, numerous epidemics attributed to pathogenic *E. coli* strains have been reported in the world [6-8]. In the East Region of Cameroon, from December 1997 to April 1998, 298 peoples have contracted a gastroenteritis epidemic caused by *E. coli* O157: H7 [9]. The contamination is made orally by ingestion of contaminated water or food.

Most physical and chemical methods used in the disinfection of bacteria-polluted water don't always yield positive results due to the fact that germs develop the ability to form complex ecosystems called biofilms, providing them with an increased resistance to disinfectants [10]. A biofilm is a set of microcolonies surrounded by a highly hydrated matrix, consisting of anionic and exopolysaccharides (EPS) [11]. The biofilms formation in the aquatic environment can be reversible or irreversible and results from interactions between the microbial and solid surface; it is often modulated by the physicochemical properties of water [12]. Several studies have been carried out on bacterial adhesion to substrates in the aquatic environment. It appears that this process also depends on the cell physiology and surface properties as well as the chemical characteristics of water and solid particle [13].

Disinfection is widely used to eliminate biofilms; unfortunately it is often ineffective [14]. Microorganisms in adhered state are often more resistant to antimicrobials than those in planktonic [15]. Numerous studies have been conducted in view of highlighting the inactivation of various waterborne pathogens by various disinfectants, including sodium hypochlorite, hydrogen peroxide, ozone, and chlorine dioxide [11]. Water disinfection by plant extracts has been suggested as a new alternative for treating water at the household level. The use of plants for therapeutic purposes is a common practice for millennia. This has been a resurgence of interest with the first research on the antibacterial properties of plant extracts in an attempt to provide a scientific basis for these empirical practices [16].

Plants have been used for the treatment of diseases all over the world before the advent of modern clinical drugs and are known to contain substances that can be used as precursors for the synthesis of useful drugs [17]. Over 50 % of modern drugs are of natural origin and most of them play an important role in the development of novel drugs in the pharmaceutical industries [18]. In Cameroon as in other developing countries, many plants used in traditional medicine against infectious diseases have been reported [19-21]. These works, achieving a microbiological and chemical screening, are of great importance. They contribute to

the orientation of people to plants with a real healing power, giving them medicines at low cost. Many studies have shown that medicinal plants contain many bioactive compounds such as flavonoids capable to act on planktonic bacteria [22]. It has also been indicated that *Eucalyptus microcorys* aqueous leaves extracts acts against planktonic bacterial metabolism [23]. Few data is available on the effect of medicinal plants extracts on bacteria adhered to organic or inorganic substrates immersed in water. The main purpose of this study was to assess in aquatic microcosm the effect of the aqueous extract of *Eucalyptus microcorys* on enteropathogenic *Escherichia coli* cells in adhered state, with respect to the cell growth phase.

2. MATERIALS AND METHODS

2.1 Harvest of *E. microcorys* and crude extract preparation

Fresh leaves of *E. microcorys* were harvested in Yaounde (Cameroon, Central Africa) and dried up at laboratory temperature ($23 \pm 2^\circ\text{C}$) for 30 days. Thereafter, leaves were ground into powder. Fifty grams of the obtained powder was mixed with 100 ml of warm distilled water and heated up to boiling temperature. Thirty minutes after the mixture was left to settle, the supernatant was removed by filtration using sieve and whattman filter membrane. The filtrate obtained constitutes the decoction. This was then dried in an oven at $45\text{-}50^\circ\text{C}$ until evaporation (3 days) [23, 24]. The obtained crystals were used to prepare the crude extract. The output of the extraction (ratio between the weight of crystals and the weight of the powder used) was 22.43% ($\pm 0.5\%$). Three ranges of extract concentration (1%, 1.5%, 2%) have been prepared using sterile NaCl (0.85%) solution. Each extract solution was then filtered firstly through sterile cotton, then using Whattman filter membrane and cellulosic membrane (porosity $0.45\ \mu\text{m}$).

2.2 Qualitative phytochemical screening

Phytochemical screening was made according to the published protocols [25, 26]. It ascertained the presence or absence of polyphenol, triterpenoids, sterols, alkaloids, saponins, gallic and catechic tannins, flavonoids, anthraquinones and anthocyanines.

2.3 Choice of substrate used and contact-angle measurements

The choice of high density polythene as adsorbent substrates was based to its relatively high resistance to shocks, high temperatures and ultra-violet rays [27, 28], its wide use in household utensils (barrels, buckets, cans) for water supply and the drinking water distribution networks. In addition, the hydrophobic character of polythene makes it a favorable adhesion of bacteria material [28].

The contact-angle measurements were made using a contact-angle goniometer with a drop-shape analysis system (Easy Drop, Kruss). For these measurements, a $3\ \mu\text{L}$ sessile droplet was placed on a substrate surface, under ambient conditions, with a computer controlled

syringe. Operation was monitored with the video capture system. Once equilibrium was reached, the image of the droplet profile was saved. The computer with the "Drop Shape Analysis" software provides the contact-angle on the surface of the drop at right and left, and calculates the average. For the very low values of the angles (lower than 25°), the image of the drop was magnified on the computer, and then photographed. The angles were then measured manually using a protractor [29].

2.4 Bacterial isolation, characterization and storage

The bacterial specie used was enteropathogenic *E. coli* (EPEC) strain. This bacterium was isolated from an urban stream in the equatorial region of Cameroon on Endo agar medium (Bio-Rad, France), using membrane filtration technique [30]. Metallic green sheen CFUs were then considered and re-growth on the standard agar medium. Its identification was then performed according to standard method [4].

Ten minutes before the experiments, 10 ml of human blood (group A+ Rh blood) were collected in Falcon tubes. Human blood cells were harvested by centrifugation at 2515,5 g for 5 minutes at 10°C, and washed three times using 10 ml of sterilized phosphate buffer (pH = 7.4). The pellets were then resuspended in 1 ml of sterilized phosphate buffer. Characterization tests were done in two steps [31]. Firstly, haemagglutination test was used to characterize the potential pathogenic strains. This characterization was done by agglutination of human red blood cells with strains of isolated *E. coli*. The test was done by emulsifying on a sterile slide one solution consisting of washed (3 times) human red blood cells (group A+ Rh blood), phosphate buffer (pH = 7.4), a pinch of α -D-mannose, and 2 to 3 colonies collected overnight from cultures of *E. coli* grown at 37°C on Mueller Hinton agar. The slide was rotated manually for 2 minutes and the result was macroscopically observed for haemagglutination. When the suspension remains consistent after 2 minutes, the test is negative and the strains is considered as the commensally. It is positive if the suspension agglutinates and the strain is considered as potentially pathogenic [4, 5].

Secondly, antisera *E. coli* (Bio-Rad, France) were used to confirm the different potential pathogenic strains noted after haemagglutination tests. The antisera *E. coli* used were the Nonavalent, Trivalent I (O111+O55+O26), Trivalent II (O86+O119+O127), Trivalent III (O125+O126+O128), and the Trivalent IV (O114+O124+O142) (Bio-Rad, France) [23].

For the preparation of bacterial stocks, a colony forming unit (CFU) of each strain from standard agar medium was inoculated into 100 ml of nutriment broth (Oxford) and incubated for 24h at 37°C. After these cells were harvested by centrifugation at 8000 rev/min for 10 min at 10°C and washed twice with NaCl (0.85%) solution. Each pellet was re-suspended in 50 ml of NaCl solution. After homogenization, 1ml of this

was then transferred into 500 ml of sterile NaCl solution (0.85%) contained in an Erlenmeyer flask and stocked.

2.5 Assessment of the cell growth phase

Three sets of 15 test tubes each containing 10 ml of sterile tryptone (Biokar) solution were used. Tubes of each set were labeled t_0 , t_2 , t_4 , t_6 , t_8 , t_{10} , t_{12} , t_{14} , t_{16} , t_{18} , t_{20} , t_{22} , t_{24} , t_{26} and t_{28} [32]. Prior to the experiments, the stock frozen vial containing cells was thawed at room temperature. The culture (300 μ l) was then transferred into 10 ml of nutrient broth (Oxford) and incubated at 37°C for 24 hours. After, cells were then harvested by centrifugation at 8000 rpm for 10 min at 10 °C and washed twice with sterile NaCl (0.85%) solution. The pellet was then re-suspended in 10 ml of sterilized solution containing NaCl (0.85%) solution. After dilution, 100 μ l was added to 100 ml of sterilized NaCl (0.85%) solution, in each of the 15 tube containing sterilized peptone solution.

Cell suspensions in the 3 tubes coded t_0 were immediately analyzed. Those in tubes coded t_2 , t_4 , t_6 ... t_{28} were incubated for 2, 4, 6... 28 hours at 37°C. The colony Forming Units (CFUs) was counted after each incubation period. The average of the CFUs after each incubation period was calculated from the results of the triplicate and the log (CFU) also calculated. The straight log (number of CFUs) curve against storage duration was plotted and then assimilated as the cell growth curve. The cell growth phases were then assessed.

2.6 Cell adhesion and detachment protocol

On the basis of previous studies, rectangular shaped (0.9 cm wide, 3.24 cm length and 0.9 cm in height) of polythene fragments with 13.28 cm² of the total surface were prepared [33] and were immersed in thirty flasks of 250 ml divided in three subgroups A, B and C. Each set, noted A_1 , A_2 A_9 for the first subgroup, B_1 , B_2 B_9 for the second subgroup and C_1 , C_2 C_9 for the third subgroup, contained 99 ml of NaCl solution (NaCl: 0.85%). Meanwhile, the controls were made and were coded A_0 , B_0 and C_0 . The experiments were conducted in static water conditions to promote the adhesion of bacterial cells to the surfaces of the plates [34]. The whole was then autoclaved.

Prior to the experiments, stocks frozen vial containing EPEC were thawed at room temperature. Then 100 μ l of the culture was transferred into test tubes containing 10 ml of nutrient broth (Oxford) and incubated at 37°C for 24 hours. Cells from a specific growth phase were then harvested by centrifugation at 8000 rpm for 10 min at 10°C and washed twice with sterile NaCl solution (0.85%). The pellets were then re-suspended in 50 ml of sterilized NaCl solution (0.85%). 1 ml of the suspension was added to 199 ml of sterilized NaCl solution (0.85 %) contained in an Erlenmeyer flask. The initial concentration of bacteria (data at $t = 0$) in each solution was adjusted to 6×10^8 CFU/ml by reading the optical density at 600 nm using a spectrophotometer (DR 2800) followed by culture on agar [34].

After each incubation duration (3h, 6h and 9h), the fragment was removed from Erlenmeyer flask A₀, B₀ and C₀ and introduced into 10 ml of sterilized NaCl solution (0.85%) as control. The fragments of each set were removed and were introduced into test tubes containing 10 ml of *Eucalyptus microcorys* extract at different concentration. Those of A₁, B₁ and C₁, after 3h of cell adhesion process in NaCl solution (0.85%), were removed and introduced during 1 hour incubation in the plant extract solution at extract concentration 1%, 1.5% and 2% respective, A₂, B₂ and C₂ were removed and introduced during 2 hours incubation in the plant extract solution at the same respective extract concentration and A₃, B₃ and C₃, were removed and introduced during 3 hours incubation in the plant extract solution at extract concentration 1%, 1.5% and 2% respective. The process was done with those removed after 6h and 9h of cell adhesion process in NaCl solution (0.85%).

After each incubation in the plant extract solution, the unhooking of adhered cells was performed by vortex agitation at 1000-1200 rpm for 10 seconds in three consecutive series of 10 ml sterilized NaCl solution (0.85%). This technique allows the unhooking of maximum adhered cells [35]. The total volume of the suspension containing the unhooked bacterial cells was 30 ml. the isolation and enumeration of unhooked cells were made by culture on Endo (Bio-Rad) agar medium, by the method of surface spreading, followed by incubation on Petri dishes at 44°C for 24 hours. The same technique of unhooking cells was used for the control substrate.

2.7 Assessment of the cell surface hydrophobicity

Enteropathogenic *E. coli* surface hydrophobicity was measured by the adhesion test on polythene using the MATH (Microbial Adhesion To Hydrocarbons) method [36] as recently described by Jain *et al.* [37]. After culture on Endo agar medium (Bio-Rad, France), cells were centrifuged at 8000 rev/min for 10 min at 10 °C, washed and resuspended at a concentration of 10⁸ CFU/ml in distilled water (pH 4.9) by reading the spectrophotometer DR 2800. The optical density (OD) of the solution was measured at 400 nm (A₀). 1 ml of this solution is introduced in 3 Erlenmeyer flasks A₁, A₁' and A₁'' containing 99 ml sterilized NaCl solution (0.85%) and parallelepipedic shaped fragments of polythene of 13.28 cm² total surface area suspended to a wire of 0.1 mm diameter. The mixture was incubated at room temperature (25°C ± 1) for 3h, 6h and 9h. After each incubation period, the fragment was removed from Erlenmeyer flask and the OD of the aqueous phase was measured (A₁, A₁' and A₁''). Concentrations of enteropathogenic *E. coli* were assessed by turbidity and expressed by measuring the optical density (OD 600

nm) on a spectrophotometer DR 2800. A density of 0.08 to 0.1 corresponded to 6 x 10⁸ CFU/ml [34]. The percentage of cells adhered to the polythene was calculated using the following formula [37]:

$$\text{Cell adhered (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

In this formula, A₀ is the OD of the solution measured at 400 nm before the substrate immersion, and A₁ is the OD of the aqueous phase measured after cell adhesion process. The more the value obtained was closer to 100 % the more enteropathogenic *E. coli* was hydrophobic. According to Rosenberg [35] and Jain *et al.* [36], the percentage of cells adhered to the polythene in this condition is closely proportional to the cell surface hydrophobicity. According to those authors, the more the value obtained is closer to 100% the more enteropathogenic *E. coli* is hydrophobic. The percentages of cells adhered were then compared to the hydrophobicity percentages [37].

2.8 Data analysis

To estimate cell adhesion kinetics (CAK) or cell adhesion speeds, a straight Ln (number of CFUs adhered/cm² of the fragment) lines was plotted. A linear line has an equation of the form $y = ax + b$. In this equation, x is the explanatory variable, a is the slope of the regression line, and b is the intercept point of the regression line on the y axis (the value of y when x = 0) [38, 39]. The slope of the straight line was divided by 3 and then was considered as the hourly cell adhesion kinetics (CAK). The Spearman correlation coefficients and comparisons amongst considered parameters were assessed. Statistical analysis was performed using SPSS version 19.0

3. RESULTS AND DISCUSSION

3.1 Phytochemical screening of *E. microcorys* extract

The phytochemical screening of *Eucalyptus microcorys* revealed the presence polyphenols (+), sterols (+), triterpenoids (+), flavonoids (++), gallic tannins (+), anthraquinones (+++), anthocyanines (+), alkaloids (+++) and saponins (++)

3.2 Contact angle values of polythene surface

The results obtained during contact angle measurements with the use of a goniometer and the DROP computer program showed that the mean value of contact angle obtained for three polythenes tested is 84.8° ± 1.1° to the left and is 84.9° ± 1.9° to the right. This contact angle variation corresponds to a surface moderately hydrophobic-hydrophilic surface [40].

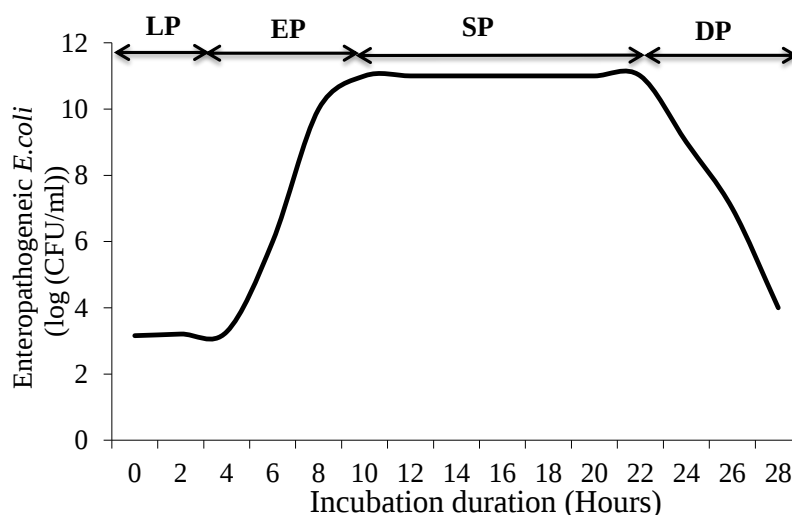


Figure 1: Growth curve of enteropathogenic *E. coli* (LP: Lag growth Phase; EP: Exponential growth Phase; SP: Stationary growth Phase; DP: Decline growth Phase).

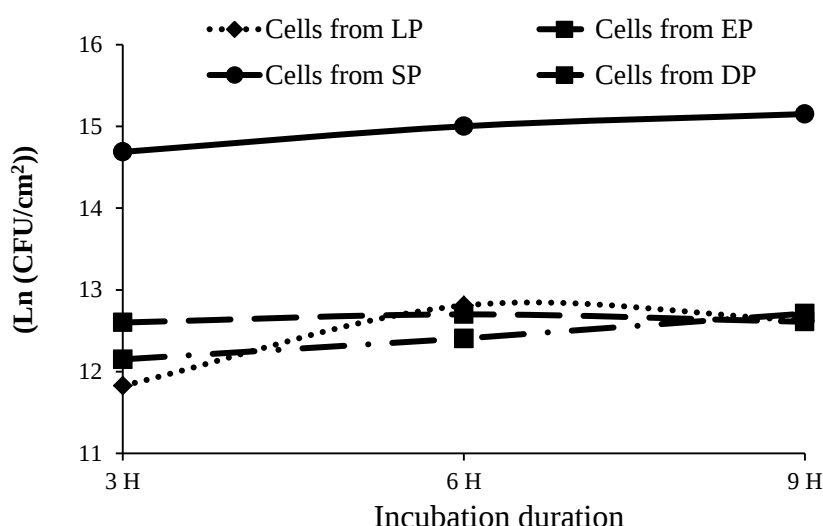


Figure 2: Number of adhered cells of enteropathogenic *E. coli* in NaCl (0.85 %) solution, cells are harvested from different growth phase (LP: Lag growth Phase; EP: Exponential growth Phase; SP: Stationary growth Phase; DP: Decline growth Phase).

3.3 Cell growth curve

The illustration of enteropathogenic *E. coli* growth rate in liquid medium non-renewed peptone show a hyperbolic curve in 4 phases. When considered t_{0h} as the initial moment, the 4 growth phases are temporally divided as follow: the lag growth phase from t_{0h} to t_{5h} , the exponential growth phase from t_{5h} to t_{11h} , the stationary growth phase from t_{11h} to t_{23h} , and the decline growth phase from t_{23h} to t_{28h} (Figure 1).

3.4 Cell adhesion kinetics

Considering the first 9 hours incubation duration of adhesion process of enteropathogenic *E. coli* to the surface of polythene substrate in NaCl solution (0.85%), the straight Ln (number of CFUs/cm²) lines against incubation duration of the form $y = ax + b$ were plotted. The slope of the straight line was then considered as cell adhesion kinetic (CAK). It appeared

that, the CAKs varied from one growth phase to another. The highest CAK was 0.133 cell adhered/cm²/h; this was registered with cells harvested from the lag growth phase. The lowest CAK 0.001 cell adhered/cm²/h was noted with cells harvested from the exponential growth phase (Table 1).

3.5 Numeration of adhered enteropathogenic *E. coli* cells in NaCl (0.85 %) solution

Figure 2 show the temporal variations of enteropathogenic *E. coli*, harvested from different growth phases, during the cell adhesion process on polythene surface in NaCl solution (0.85%). The bacterial abundances on polythene control, expressed in Napierian Logarithmic units (CFUs/cm²) ranged from 11.8 to 12.8 from 12.6 to 12.7, 14.7 to 15.2 and

12.2 to 12.7 respectively at the lag, the exponential, the stationary and the decline growth phases (Figure 2).

3.6 Percentage of cells of enteropathogenic *E. coli* adhered

In sterilized NaCl solution (0.85 %), the percentage of adhered enteropathogenic *E. coli* ranged from 67.78 to 72.89% for cells coming from lag growth phase; 80.11 to 83.24% for cells coming from exponential growth phase; 79.49 to 82.62% for bacteria coming from

stationary growth phase and from 47.12 to 50.92% from cells coming from decline growth phase. These percentages relatively increased with incubation duration. For the whole experiment, the highest values were recorded at the exponential growth phase and the lowest recorded from cell harvested from decline growth phase (Figure 3).

Table 1: Value of cell adhesion kinetics (and regression coefficient) of enteropathogenic *E. coli* with respect to growth phases.

Cellular growth phases	Adhesion kinetics (Cell adhered/cm ² /h)
Lag	0.133 (0.586)
Exponential	0.001 (0.006)
Stationary	0.077 (0.960)
Decline	0.093 (0.997)

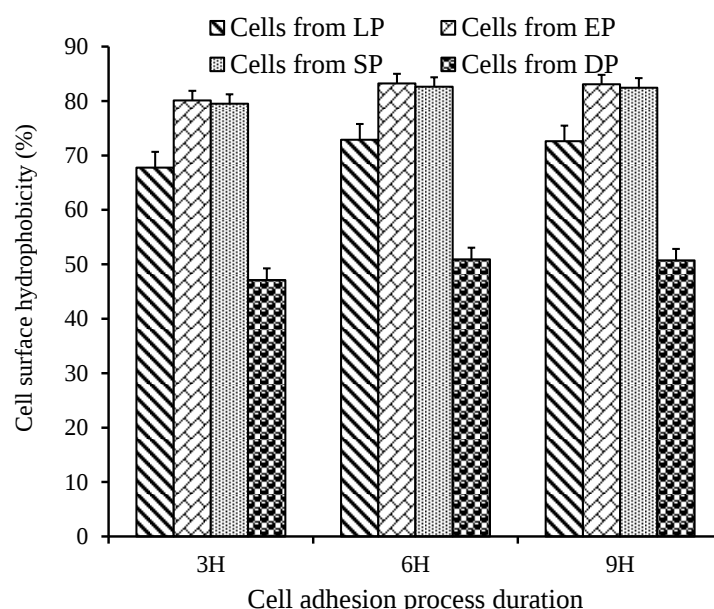


Figure 3: Physicochemical surface properties of adhered enteropathogenic *E. coli* cells harvested from Lag growth Phase (LP), Exponential growth Phase (EP), Stationary growth Phase (SP), and Decline growth Phase (DP).

3.7 Immersion of cells adhered in the plant extracts solution and cells detached assessment

Substrates with cells adhered were immersed in *Eucalyptus microcorys* extract solutions at different concentrations (1%, 1.5% and 2%). The cells detachment was then observed during incubation periods (1h, 2h and 3h). The detached cells, after withdrawal of substrate contained adhered cells harvested from each growth phase, fluctuated with to the incubation duration and the extract concentration (Figure 4). For cells stemming from lag growth phase, the highest values of abundance of detached cell were 8.71, 8.72 and 8.61 (Ln (CFU/cm²)) after 1h, 2h and 3h incubation respectively. This was noted after 3 hours duration of cell adhesion process (DCAP) and at the concentration of 2%. After 6h of DCAP and for the whole extract concentrations, detached cell abundances ranged from 7.15 to 9.29, from 7.45 to 9.43 and from 9.23 to 9.81 (Ln (CFU/cm²)) respectively after

1h, 2h and 3h. After 9h of DCAP, the detached cell abundances ranged from 10.25 to 10.43, from 9.77 to 10.31 and from 8.41 to 10.43 (Ln (CFU/cm²)) respectively after 1h, 2h and 3h.

For cells coming from exponential growth phase, the higher value of detached cell abundances after 1h incubation was 10.54 (Ln (CFU/cm²)). It was registered after 3 hours of DCAP, at the extract concentration 2%. After 2h and 3h of incubation, the highest values were respectively 9.29 and 10.24 (Ln (CFU/cm²)). For the whole extract concentrations after 6h of DCAP, detached cell abundances ranged from 10.18 to 10.77, from 8.42 to 10.32 and from 8.38 to 10.25 (Ln (CFU/cm²)) respectively after 1h, 2h and 3h. After 9h of DCAP, they ranged from 9.44 to 10.11, from 8.33 to 8.62 (and from 7.97 to 8.95 (Ln (CFU/cm²)) respectively after 1h, 2h and 3h incubation in the plant extract solution.

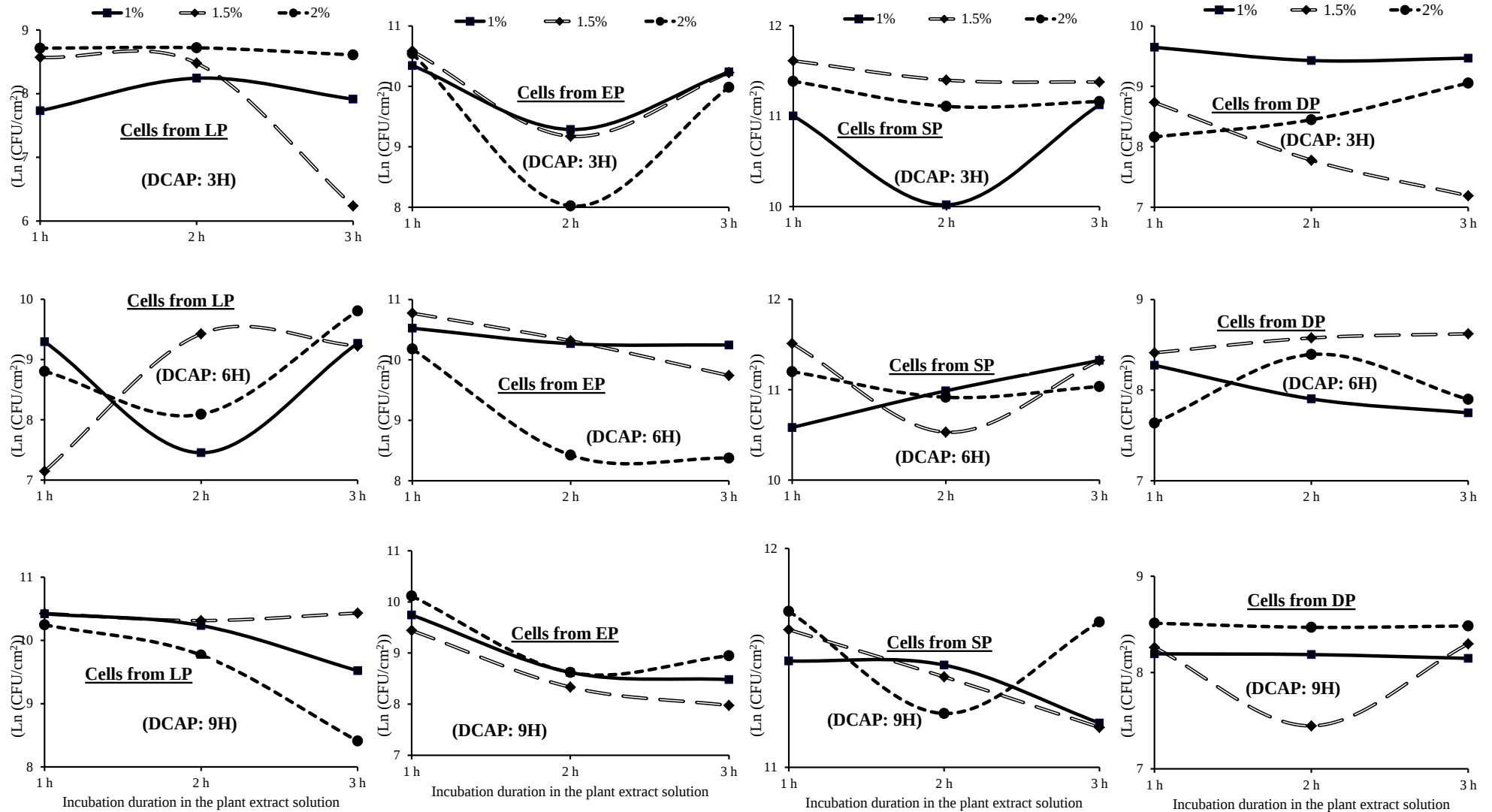


Figure 4: Temporal variation of the abundance of detached enteropathogenic *E. coli* cells after the living in the plant extract solution at different concentrations (1 %, 1.5 % and 2 %), cells harvested from Lag growth Phase (LP), Exponential growth Phase (EP), Stationary growth Phase (SP), and from Decline growth Phase (DP), after the duration of cell adhesion process (DCAP) of 3H, 6H and 9H.

For cells harvested from stationary growth phase and after 3 hours DCAP, the detached cell abundances ranged from 11.00 to 11.39, from 10.02 to 11.40 and from 11.12 to 11.38 (Ln (CFU/cm²)) respectively after 1h, 2h and 3h incubation. After 6h DCAP, they ranged from 10.58 to 11.51, from 10.53 to 10.99 and from 11.03 to 11.32 (Ln (CFU/cm²)) respectively after 1h, 2h and 3h incubation in the plant extract solution. After 9h of DCAP they ranged from 11.49 to 11.7, from 11.25 to 11.47 and from 11.18 to 11.66 (Ln (CFU/cm²)) respectively after 1h, 2h and 3h incubation in the plant extract solution.

The lowest count of cell detached was noted after 3 hours incubation in the plant extract solution at concentration 1.5% when cells coming from decline growth phase. After 6h of DCAP, the detached cell abundances fluctuated between 7.64 and 8.41, 7.90 and 8.58 and between 7.75 and 8.62 (Ln (CFU/cm²)) respectively after 1h, 2h and 3h incubation. Variations of the detached cell abundances were also observed after 9h of DCAP (Figure 4).

Table 2. Percentage of detached cell (% D) after 1h, 2h or 3h incubation in the plant extract solution of the concentration 1 %, 1.5 % and 2 %, after each Duration of cell adhesion process (DCAP: 3H, 6H and 9H) in NaCl solution (NaCl 0.85 %) when cells were from each growth phase.

Cell growth phase and duration of cell adhesion process		Percentage of detached cell (% D) after 1h, 2h or 3h incubation in the plant extract solution of the concentration 1 %, 1.5 % and 2 %								
Cell growth phase	Duration of cell adhesion process	1%			1.5%			2%		
		1h	2h	3h	1h	2h	3h	1h	2h	3h
Lag	3H	1.7	2.8	2.0	3.9	3.5	0.4	4.4	4.5	4.0
	6H	2.9	0.5	2.9	0.4	3.4	2.8	1.8	0.9	4.9
	9H	11.0	9.2	4.5	11.1	9.9	11.2	9.3	5.7	1.5
Exp.	3H	10.5	3.6	9.4	13.3	9.3	62.8	12.7	1.0	7.3
	6H	11.4	8.8	8.6	14.6	5.2	68.8	8.1	1.4	1.3
	9H	5.7	1.9	1.6	4.2	0.9	12.9	8.3	1.9	2.6
Stat.	3H	33.3	12.4	37.6	4.6	3.7	48.5	3.7	2.8	2.9
	6H	16.0	24.0	33.6	3.1	2.5	33.5	2.2	1.7	1.9
	9H	34.0	33.4	25.6	3.0	1.9	25.1	2.9	1.3	1.8
Decl.	3H	55.6	44.9	48.3	3.3	0.7	9.7	1.9	2.5	4.5
	6H	21.3	14.7	12.6	1.8	2.3	30.2	0.9	1.8	1.1
	9H	14.5	14.4	13.8	1.2	1.2	16.0	1.5	1.8	4.8

Lag.: lag growth phase ; Exp.: exponential growth phase ; Stat.: stationary growth phase ; Decl.: decline growth phase

3.8 Percentage of detached cells

Different percentages of detached cells, after incubation with *Eucalyptus microcorys* extract solution at different considered concentrations are summarized in Table 2. When the bacteria cells were harvested from the lag growth phase, the percentage of detached cells fluctuated between 1.7 and 11, 0.5 and 9.2, and between 2.0 and 4.5% after 1h, 2h and 3h incubation respectively at concentration 1% of extract solution. At the concentration 1.5%, it fluctuated between 0.4 and 11.1, 3.4 and 9.9, and between 0.4 and 11.2% after 1h, 2h and 3h incubation respectively. At the concentration 2%, it fluctuated between 1.8 and 9.3, 0.9 and 5.7, and between 1.5 and 4.9% after 1h, 2h and 3h incubation also.

From cells coming from exponential growth phase, the highest counts of cells detached were 11.4, 8.8 and 9.4% after 1h, 2h and 3h of incubation at the extract concentration solution of 1%. They were 14.6, 9.3 and 68.8% after the same incubation periods at concentration 1.5%, and were 12.7, 1.9 and 7.3% in the plant extract solution at concentration of 2%. Variation in the abundances of cells detached was also noted when cells used for experiments were harvested from stationary phase. In most cases, the highest percentages of detached cells are observed with cells

coming from exponential, stationary and decline growth phases. The highest percentages of detached cells (above 50%) were noted for solution of extract at 1% concentration with cells coming from decline growth phase (Table 2).

3.9 Relationships between considered parameters

Spearman correlation coefficients between the abundances of adhered cells when the polythene was immersed in NaCl solution (0.85%) and the abundances of detached cells after living in the plant extract solution at each extract concentration were calculated. A positive and significant relationship ($P < 0.01$) was noted between these two parameters for each of the three extract concentrations. The Kruskal-Wallis test was used to compare the abundances of detached cells harvested at different cell growth stages. Differences ($P < 0.01$) were statistically significant even when we considered each of the three extract concentrations.

The growth curve of enteropathogenic *E. coli* obtained showed a lag phase from 0 and 5 hours, during which the bacteria adapt to a fresh medium, an exponential growth phase from the 5th to the 11th hour, a stationary growth phase from the 11th to the 23th hour, and a decline phase or phase of death cells which starts from the 23th hour after incubation duration. When cells are

transfer in an environment where new biosynthesis are required, they take time called lag time, for the implementation of enzymes that intervene in these syntheses [41]. Use of nutrient and cell duplication occurs during the exponential growth period. During the stationary growth phase, no significant temporal variation is observed in the cells abundance. However, in the stationary growth phase many cellular functions, including energy metabolism and biosynthesis, continue. The decline phase is the period when all food resources are exhausted: toxic metabolite accumulates; the concentration of viable organisms decreases; the cellular lysis by endogenous proteolytic enzymes can also occur. However, a low growth rate could still be detected following the releasing of substances during lysis (cryptic growth) [42].

The use of enteropathogenic *E. coli* cells harvested at different cellular growth phases, has achieved the adsorption tests on polythene substrate. It has been noted that cells adhesion depends on the physiological state of the bacteria. The evaluation of cell adhesion speeds showed a kinetic of 0.133 cell adhered/cm²/h for cells harvested from the lag growth phase, 0.001 cell adhered/cm²/h for cells harvested from the exponential growth phase, 0.077 cell adhered/cm²/h for cells harvested from the stationary growth phase and 0.077 cell adhered/cm²/h for cells harvested from the decline growth phase. The variation of these cell adhesion speeds may be influenced by the mechanism of reversible and irreversible adhesion and also could be explained by changes in cell physiology [11, 43].

Cells adhesion to a solid particle uses different types of interaction depending on the nature of the substrate to colonize. It also depends on the reversibility of the initial stage of the process due to the adsorption-desorption phenomena [44]. The bacterial adhesion to substrates involves two main steps: reversible adhesion and irreversible adhesion [45]. The reversible adhesion is governed by physico-chemical interactions of type Van der Waals and Lewis acid-base [28]. The irreversible adhesion is slower than the previous one, the irreversibility of the membership using the bacterial metabolism step. The hydrophobicity of the surfaces of microorganisms is generally regarded as one factor among others in the adhesion phenomenon.

The physicochemical properties of the surface hydrophobicity of the microorganisms are related to the chemical composition of the outer membrane and the conformations of the resulting molecular structures. This hydrophobicity could influence the capacity of cells to adhere on polythene. For authors the hydrophobicity of the bacterial surface is the key parameter that governs bacterial adhesion to inert supports [46, 47]. The results obtained by the MATH method showed that enteropathogenic *E. coli* is more hydrophobic when the cells comes from exponential and stationary growth phases. The cell surface hydrophobicity increased with incubation periods. The minimum value of the hydrophobicity percentage was

recorded with cells harvested from the decline growth phase of with values ranged between 47.12 to 50.92%. The moderately hydrophilic-hydrophobic nature of polythene can also influence the adhesion of the bacterial cells. The influence of the solid surface's hydrophobicity on the adhesion has been indicated [48].

The bacterial adhesion process to substrates is based on physicochemical properties of the latter [28]. The hydrophobic - hydrophilic character of substrate is reflected by measuring the contact angle. Surfaces with contact angles greater than 90° are designated as hydrophobic. When 30° < θ < 90°: the support is moderately hydrophobic and hydrophilic [40]. A contact angle of 73.3° on polythene was found by other authors [29, 49]. It has been noted that *Aureobasidium pullulans* adheres more on media with increasing hydrophobicity [50]. These results are similar with those on *Enterococcus faecalis* with a higher adhesion to the silicon compared to the glass [50, 51].

During the present study, it has been noted that the abundance of enteropathogenic *E. coli* attached to the polythene varies when the latter is immersed in the *Eucalyptus microcorys* extract solution. The cell's detachment has been observed. The extract causes cell's detachment and this depends on the incubation duration of substrate as well as on the concentration of the plant extracts. The cell detachment observed could be due to the presence of some secondary metabolites in aqueous *Eucalyptus microcorys* extract which can disrupt physicochemical surface characteristics of enteropathogenic *E. coli*.

In this study, the phytochemical of the plant extract revealed the presence of several organic compounds as polyphenol, triterpenoids, sterols, alkaloids, saponins and gallic tannins, flavonoids, anthraquinones and anthocyanines. The presence of these compounds has been reported in other studies [23, 52]. Polyphenols in the plant extract would be a form of stress and would probably lack the bacteria of their protective glycocalyx causing disruption and dislodging biofilm bacteria on the surface of polythene substrate. These secondary metabolites would cause of the rupture of hydrogen bonds within the exopolysaccharide protective matrix secreted by bacterial cells. However, the rates of detached cells are sometimes lower to 15%. This relative low rate could be due to the exopolymer covering bacteria that creates a concentration gradient so that the leakage of the protective layer is not complete. Thus, only the bacterial cells located at certain distance of the solid are achieved dislodged. Also, some bacteria have specific plasmid genes that encode virulence factors (the type IV pili, adhesins, and toxins) which play an important role in the cell adhesion process [53]. They allow the interconnection of bacteria in micro-colonies promoting their stabilization, which could cause resistance to the cell detachment.

It has also been noted that the cellular growth phase from which cells used in the experiments were harvested impacted the abundance the cell adhesion detachment process. A positive and significant correlation ($P < 0.01$) between the cell abundances adhered to substrate control and the abundances of detached cells has been noted. The abundances of detached cells are positively and significantly correlated with the cellular growth phases. Significant difference ($P < 0.01$) between adhered and detached cells abundances after living in the plant extract solution. All this could be due to the accession rate that varies depending on cellular growth phase that bacteria come from, during the adsorption test. The exponential growth phase results in a high cell activity whereas the stationary growth phase has a slowing down of this activity, resulting in chemical modifications to the surface of the cell [54]. The stability and the reactivity of secondary metabolites like flavonoids are commonly linked to their molecular structures [55]. The reactivity of flavonoids dissolved in water leads to the molecular instability and this may vary with changes in environmental properties. The amount of detached cells seems to vary with the cell growth phase as well as with the concentration of the plant extract and incubation duration. This variation may also depend on the potential of secondary metabolites which are present. Each of them may act solely or in synergetic with others [56].

4. CONCLUSION

Bacterial adhesion process is well reported to exhibit increased resistance to antimicrobial agents compared with their planktonic counterparts, due in part to the physical barrier that they create. The use of the extract of *Eucalyptus microcorys* seems to have a significant impact on this barrier. Causing detachment of cells to substrate, they are more exposed to secondary metabolites that have a relative antibacterial activity. The polyphenols, present in the extract plant, would act by permeabilization of the exopolymere secreted at the formation of the biofilm. The use of the bioactive compounds of the *Eucalyptus microcorys* extract can be an alternative process, therefore, in the struggle against the processes of adhesion to the support of the bacteria or against the present biofilms in the ways of pipeline, and also in the bacterial cell inhibition.

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