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Cocoa vs Pathogenic Bacteria of Human and Animal Concern

Paola Del Serrone[1]*, Salvatore Claps[1] and Marcello Nicoletti[2]

[1]Council for Agricultural Research and Economics CREA, Research Centre for Zootechnics and Aquaculture CREA ZA, Italy

[2]Department Environmental Biology, University of Rome, Sapienza, Rome, Italy

***Corresponding Author:** Paola Del Serrone, Council for Agricultural Research and Economics CREA, Research Centre for Zootechnics and Aquaculture CREA ZA, Va Salaria 31 00015 Monterotondo RM, Italy. E-Mail: paola.delserrone@crea.gov.it

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Abstract

Cocoa seeds are nowadays the centre of an important debate on their potential claims and health beneficial properties. Cocoa boasts a unique position among plant drugs containing xanthine alkaloids, being a mixture of pharmacological and nutritional aspects. The scientific world is requested to clarify its real biological activities and validate derived products. We report evidences of activity of cocoa against some pathogenic bacteria of animal and human relevance. The report is fuelled by the necessity of further antibiotic agents, in consideration of the multi-resistance phenomenon performed by microorganisms.

Keywords: *Antibiofilm Agent; Analogue Receptor; Cocoa Products; Melanoidins; Enteropathogenic Bacteria; Epicatechins*

Abbreviations

ACSSuT Resistance to Ampicillin, Chloramphenicol, Streptomycin, Sulphonamides, and Tetracyclines; AOAC: Association of Official Analytical Chemists International; ATCC®: American Type Culture Collection; Cdt a, B, C: Cytolethal Distending Toxin; BIC: Biofilm Inhibitory Concentration; BSA: Bovine Serum Albumin; Cia: *Campylobacter* Invasive Antigens; Csar: Gene/Carbon Storage Regulator Protein; EPEC: Enteropathogenic *Escherichia coli;* Hlya: A-Haemolysin; UPEC: Uropathogenic *E. coli;* CNF1: Cytotoxic Necrotizing Factor 1; ISO: International Organization for Standardization; OD: Optical Density; Nleh1: Non-LEE-Encoded Effector H1; Nleh2 Non-LEE-Encoded Effector H2; PCR: Polymerase Chain Reaction; mPCR: Multiplex Polymerase Chain Reaction; PAI: Pathogenicity Island; PBS: Phosphate-Buffered Saline; QS: Quorum Sensing; T3SS: Type Three Secretion System; UTI: Urinary Tract Infection

Introduction

This paper is part of a study regarding the concernments of cocoa and its derived products [1]. Cocoa (*Theobroma cocoa* L., Sterculiaceae) is increasing its importance in human consume for several solid reasons, including new forms and claims. The potentiality of cocoa beans are still source of interest and allure. Cocoa is obtained by cultivations spread in tropical countries and pertaining to tree varieties, i.e. *Forastero*, *Trinitario* and *Criollo*, but a myriad of cross breeding, cultivars are spread everywhere. *Forastero* cover 95% of the world production of cocoa even though the highest quality of cocoa is from the *Criollo* variety and from the native *Forastero* variety of Ecuador (*Arriba*). The cocoa tree sprouts two harvests of cocoa pods per year. There 20 up to 40 purple cocoa beans encased in a sweet white pulp can be found within each pod. The raw material for the production of chocolate and powder cocoa is fermented dry cocoa beans processed in the countries of origin [2,3]. The cocoa pre-processing steps (harvest, breaking, fermentation, drying and winnowing to separate the shell from the nib) are important to ensure high quality of beans. In addition, the cocoa variety and its geographical origin are factors affecting taste and flavour of the cocoa products [4]. Other variations are generated in the post-harvesting treatments [5].

Among plants containing xanthine alkaloids, cocoa merits a special place. Coffee, tea, cola, mate are essentially consumed for their stimulant effects, whereas cocoa, and the derived products, are highly appreciated for many additional features, including the nutritional importance [6]. Eating cocoa products, like chocolate, means a special appealing special mixture of alimentation, satisfaction and excitation. Recently, the skylines of cocoa enlarged in nutraceuticals and related products [7]. Therefore, besides the traditional forms like chocolate, new derived products are appearing on the market, like functional foods. In these cases, cocoa is added to microalgae, cereals and others, with claims related to physiological properties, focused on health benefits and the antioxidant effects, among many others. As a matter of fact, several studies are in progress to exploit the potentialities of utilization of cocoa beans.

Additional features of cocoa must be derived from the complexity and richness in chemical composition of cocoa beans. However, chemistry in cocoa beans is subjected to large variability, which is the result of the type of utilized raw material. As evidenced by us in three cultivations in Cameroon, and confirmed by other studies, cocoa beans chemistry is subjected to relevant variations according to the environmental conditions [1].

Besides the genetic and environmental factors, post-harvesting elements must be carefully considered for their great influence in chemical composition of cocoa beans. Fresh cocoa beans fermentation consists in a microbial fermentation of the pulp surrounding the beans. Microbial populations, mainly of yeast (*Kloeckera* and *Saccharomyces* spp.), of lactic acid and acetic-acid bacteria (*Lactobacillus*, *Bacillus*, *Pediococcus*, *Acetobacter* and *Gluconobacter* spp.), spontaneously induce the fermentation. In such way, a wide range of metabolic end-products is produced, like alcohols and organic acids, changing radically the initial composition. Fully fermented cocoa beans show brown colour related to initial polyphenol content and enzymatic browning catalysed by polyphenol oxidase [8]. The additional steps consist in drying of the fermented cocoa beans and subsequent roasting of the fermented dry cocoa beans and/or removal of most of fatty acids.

Roasting is the most important technological operation in cocoa beans processing. Heat processed food as consequence of the Maillard reaction contains new compounds with reduction of other as sugars and proteins that originates aroma and colour formations, but also production of new components, like the melanoidins: polymeric high molecular weight, brown-coloured compounds [9]. Many beneficial effects have been associated to melanoidins, such as antioxidant, antimicrobial, anti-inflammatory, antihypertensive and prebiotic activity, among others. Biological properties of food melanoidins make these products and their by-products containing melanoidins to be considered as potential food ingredients for healthier and tasty foods [10].

Furthermore, the presence of other active metabolites was ascertained in cocoa products. Several studies showed that cocoa powder having antioxidants properties, higher then red wine or green tea [11], contain high quantities of polyphenolic compounds, like epicatechins, that are important because they offer potential cardiovascular health benefits, antioxidant protections and help balance cholesterol in the body. High temperatures during the cocoa bean roasting and alkalization of the cocoa powder are the main factors inducing the epimerization reaction which converts (-)-epicatechin to (-)-catechin and their polymers, up to decapolymers, as evidenced by analytical analyses [1,12,13].

In consideration of these peculiarities, and the increasing claims concerning cocoa and derived products, the antibacterial activity of some cocoa beans was tested against a series of important pathogens [14]. The aim of this study was to verify the presence of components of different cocoa beans and derived products for gut health-promoting adhesion of enteropathogenic bacteria, so interfering with bacterial adhesion mechanisms as bacterial receptor analogues [15], as well as anti-biofilm agents. Therefore, this is a study to validate further utilizations of cocoa, and in particular for production of antimicrobial agents, or validate its utilization in nutraceuticals and functional foods

Materials and Methods

Theobroma cocoa samples

Ten commercial cocoa products were considered. Six were roasted cocoa beans (CREA1-CREA6), three were powder cocoa products (CREA7 - CREA9), and one, fermented dried cocoa beans, was from the UNIROMA1 cocoa sample collection. They were from different countries and from different cocoa varieties (Table 1).

Number	Sample	Variety	Origin	Product
1	CREA 1	*Forastero*	Mexico	rcb
2	CREA 2	*Forastero*	Perù	rcb
3	CREA 3	*Criollo*	Venezuela	rcb
4	CREA 4	*Trinitario*	Ghana	rcb
5	CREA 5	*Criollo*	Ghana	rcb
6	CREA 6	*Forastero*	Sierra Leone	rcb
7	UNIROMA1	Unknown	Cameron	fdcb
8	CREA 7	*Forastero*	Venezuela	pc
9	CREA 8	*Criollo*	Costa d'avorio	pc
10	CREA 9	*Forastero*	Perù	pc

Table 1: *Cocoa products tested in the experiment. They are from cocoa sample collections of CREA and UNIROMA1. They belong to different varieties, geographical origin and they are at different pre-processing or processing steps as follows: rcb: Roasted Cocoa Beans; fdcb: Fermented Dried Cocoa Beans; pc: Powder Cocoa Products.*

Bacterial strains and growth conditions

The bacteria, namely, *Escherichia coli* EPEC and *E. coli* UPEC strains, *Salmonella enterica* and *Campylobacter jejuni*, were considered in the experiment.

The EPEC tested NLK99-3∗ *E. coli* strain was from calve faeces. It is resistant to the antibiotic ciprofloxacin [16].

E. coli UPEC, *Campylobacter* spp. and *Salmonella* spp. were isolated from stable wastewater. Chromogenic RAPID' *E. coli* 2 Medium (BIO-RAD Laboratories Inc. Mi Italy), a selective chromogenic agar, was used for detection and enumeration of *E. coli* and coliforms at 37°C and 44°C without further confirmation in 18 - 24 hr at 41°C. *Salmonella* spp. isolates were checked on Chromogenic RAPID' *Salmonella* Medium (BIO-RAD Laboratories Inc. Mi Italy).

This is a sensitive chromogenic agar used for the detection and enumeration of *Salmonella* spp. from environmental samples in 24 hr. While, *Campylobacter* spp. were isolated on Chromogenic RAPID' *Campylobacter* Medium (BIO-RAD Laboratories Inc. Mi Italy), that is a highly selective medium at 42°C for 24 - 48 hr in microaerophilic atmosphere. All media are according to the ISO 16140 standard and validated by AOAC as summarized in table 2.

Bacteria	Growth Medium	Temperature (°C)	Growth period (h)
Escherichia coli	RAPID' *E. coli*2 Medium; Minca and 1% Iso Vitalex Agar/Broth (100 rpm)	44 37	24 18
Salmonella spp.	RAPID' *Salmonella* Medium Nutrient Agar/Broth	41 37	24 24
Campylobacter spp.	RAPID' *Campylobacter* Medium Microaerophilic conditions (3% - 5% O_2 -10% CO_2) Mueller Hinton Broth	37 42	24 48

Table 2: *Media and growth conditions used to isolate bacteria from stable wastewater.*

Bacterial cultures for antibacterial testing were prepared by picking colony from 24-hour-old plates and suspending them in the media as in table 2 (5 mL). For antibacterial activity assay, 1 mL of each culture was diluted to 10^5 - 10^6 CFU/mL.

The cultivation/assay medium for *E. coli* was Minca + 1% Iso Vitalex Agar/Broth (Becton Dickinson. Microbiological Systems, Cockeysville, MD, USA).

The following reference strains were considered: *E. coli* ATCC® 51813™, *S. enterica* ATCC® 13076™, *C. jejuni* ATCC® 33291™. The reference strains were grew on media and at the growth conditions as reported on products sheets.

Molecular identification and characterization of bacterial strains

DNA Extraction

Genomic DNA of the bacterial isolates was extracted from 1 mL (10^6 CFU/mL) of each liquid media, where one colony of each bacterial strain was grew as described above, using a DNA extraction kit (Roche, MI, Italy) according to the manufacturer's instruction. The supernatant containing the DNA was transferred to a clean tube and stored at -20°C until used for PCR

DNA Amplification

Two primer pairs that amplify specific *E. coli* 16S rRNA sequences and fourteen primer pairs that specifically amplify target gene coding for virulence factors (adhesins and toxins) were employed to characterize the *E. coli* isolates as described in a previous work [16].

Three primers pairs were considered which target Inv-A gene, which is specific for *Salmonella* spp., IE 1, specific for *S. enteritidis* and Flic-C specific for *S. typhimurium* [17].

The primer pair lpxAF0301 and lpxARKK2m were used for specific detection of *Campylobacter* genus. Forward primers complementary to the lpxA nucleotide sequence of *C. coli* (lpxAC), *C. jejuni* (lpxAC. Jejuni), *C. lari* (lpxAC. Lari), and *C. upsaliensis* (lpxAC. Upsaliensis) were used in combination with the reverse primer lpxARKK2m, for detection of *Campylobacter* species by multiplex PCR according to Girgis., *et al* [18].

The positive and negative control, to exclude any source of contamination, were considered. The PCR products (5 µL) were analyzed by 2% or 3% agarose gel (Sigma, MI, Italy) electrophoresis buffered in 0.5x TBE (TBE buffer: 90 mM tris(hydroxymethyl)aminomethane, 90 mM boric acid, and 3mM ethylenediaminetetraacetate Na salt, pH 8.3, Sigma-Aldrich, Milano, Italy) against a 50 bp, 100 bp, and 1 Kb ladder used as size marker (Invitrogen, Milano, Italia). Then, they were visualized with 254 nm UV light (Fotodine 3-3102 Celbio, Milano, Italy) after gel staining with GelGreen™ (Biotium, MI, Italy) (Table 3).

Bacterium	Target genes	Primer pair sequence (5'-3')	Amplicon (bp)	Reference
Escherichia coli	E16SI	F CCCCCTGGACGAAGACTCAC R ACCGCTGGCAACAAAGGATA	401	16
	E16SII	F AGAGTTTGATGGCTCAG R GGACTACCAGGGTATCTAAT	798	16
E. coli EPEC	Sta	F TCC GTG AAA CAA CAT GAC GG R ATA ACA TCC AGC ACA GGC AG	244	16
E. coli UPEC	hlyA	F AGCTGCAAGTGCGGGTCTG R TACGGGTTATGCCTGCAAGTTCAC	569	16
Salmonella spp.	InvA	F CGG TGG TTT TAA GCG TAC TCT T R CGA ATA TGC TCC ACA AGG TTA	796	17
S. enteritidis	IE-1	F AGT GCC ATA CTT TTA ATG AC R ACT ATG TCG ATA CGG TGG G	316	17
Campylobacter spp.	Gene 0301	F CTT AAA GCN ATG ATA GTR GAT AAR R CAA TCA TGD GCD ATA TGA SAA TAG GCC AT	521	18
C. jejunii	LipidA (lpxA) gene	F ACA ACT TGG TGA CGA TGT TGT A R CAA TCA TGD GCD ATA TGA AAA TAG GCC AT	331	18

Table 3: *Species-specific primer pairs (column 3) against target genes of Escherichia coli, E. coli EPEC, E. coli UPEC, Salmonella spp., S. enteritidis, Campylobacter spp., and C. jejunii (column 2) used in PCR and mPCR for the identification and the characterization of the enteropathogenic bacteria isolated from stable wastewater and used in the biological activity assays. The expected amplicon's size and the references for the PCR and mPCR reaction's mixtures and conditions are in column 4 and 5.*

Biological activity assays

Adhesion test

The cocoa products were applied as coating materials in the adhesion test along with BSA as a control. Microplate adhesion experiments were carried out as described by Becker., *et al.* [19], Becker and Galletti [20]. The test products were diluted in PBS buffer to a final concentration of 1% (w/v). After sonicating and centrifuging, the supernatants were pipetted into a polystyrene microplate (350 µL/well; high-binding Microlon F plate 655092 Greiner Bio-One B.V., RM, Italy) using them as coating.

The micro plates were then incubated overnight at 4°C. Non-coated wells were included as negative controls in each plate. After that, the plates were washed with 350 µL PBS buffer to remove non-binding coating material. Blocking of the microplates was done by incubating the wells with 350 µL of 1% BSA in PBS (w/v) that contained 0.5% sodium azide at 4°C for 1h. Then, plates were washed twice with 300 µL of PBS. Bacteria that has been grown, washed and suspended in PBS were added into the microplate wells (300 µL/well) and allowed to adhere at room temperature for 30 minutes.

Afterwards, the wells were washed three times with 300 µL of PBS to remove non-adherent bacteria. Then the wells were filled with 300 µL growth medium (BHI or Minca).

The control wells were filled with 300 µL of a ten-fold dilution series in growth medium (BHI or Minca) with a known amount of each test bacterium.

Then, the microplate was placed in a microplate reader (SpectraMax 340; Molecular Devices Ltd., Wokingham, United Kingdom), incubated at 37°C, and shaken at medium intensity for 3 sec, prior to every reading. The OD was determined at a wavelength of 650 nm every 15 min during 24 hours. All readings done in two independent assays and in quadruplicate per microplate. The data generated by the photometer software (SoftMaxPro 2.2.1.; Molecular Devices Ltd., Wokingham, United Kingdom) were processed by non-linear regression analysis employing the Boltzmann sigmoidal equation to describe the kinetics of bacterial growth:

$$Y=Bottom + (Top-Bottom)/(1+exp((V50-X)/Slope)).$$

In this equation, V50 (t) is the time at which half of the maximal yield has been reached. Analyses of variance were performed using GenStat (VSN International Ltd., Hemel Hempstead, UK). The V50s were also converted to adhering cell numbers of the test bacterium according to Becker., *et al.* [19,20], using the V50s of the ten-fold dilution series of each tested bacterium.

Biofilm formation assay

Biofilm Formation Assay performed in 24-Well Polystyrene Plate. The effect of CAB extracts on biofilm formation was done in 24-well polystyrene plates. Briefly, overnight cultures of the test organisms (1%) were inoculated with 1 mL of fresh TSB in the presence (treated) and absence (untreated) with cocoa powder at different dilutions (100 µL, 10 µL, 1 µL, 0.1 µL). The plates were incubated for 24h at 37° C. After incubation, the plates were washed with sterile phosphate buffered saline (PBS) to remove the free cells and allowed to air dry before being stained. The biofilms were stained with 0.4% crystal violet solution (w/v) for 5 minutes. Subsequently the unstained dye was discarded, and the wells were rinsed twice with deionized water and then allowed to dry. Finally, 1 mL of absolute ethanol was added in each well. The optical density was determined at 570 nm, and percentage of biofilm inhibition was calculated using the following formula:

$$\text{Percentage (\%) of inhibition} = [(\text{Control OD 570 nm} - \text{Test OD 570 nm})/\text{Control OD 570 nm}] \times 100.$$

The biofilm inhibitory percentage reduction was determined as the lowest growth that produced visible disruption of biofilm formation and a significant reduction in the readings when compared with that of the control wells at OD 570 nm. Wells containing medium and extract were used as blanks.

Results and Discussion

Microbiological and molecular biology analyses let the identification of pathogenic *E. coli* EPEC and UPEC, *Salmonella enteritidis* and *Campylobacter jejuni* isolates from stable water.

The high specificity detection on RAPID' *E. coli*2 Medium is based on β-D-Glucuronidase (GLUC) and β-D-Galactosidase (GAL) activities. *E. coli* (GAL+/GLUC+) was revealed since it forms violet to pink colonies, while other coliforms (GAL+/GLUC-) form blue to green colonies.

RAPID' *Salmonella* Medium let the identification of *Salmonella* spp. which takes the form of easily identifiable typical magenta colonies, due to the activity of a C8 esterase. The resulting colonies developed on RAPID' *Campylobacter* Medium were brick-red coloured. The genus and species-specific PCR and mPCR yielded amplicons of expected sizes as listed in table 3 according with those reported in literature.

Among pathogenic enterobacteria, *E. coli* changes into a pathogen by acquisition of genetic elements called PAIs. The PAI virulence genes of EPEC act when the bacterium adhere to a host gut cell [21]. EPEC is an intestinal attaching and effacing pathogen that utilizes a T3SS for the delivery of anti-inflammatory effector molecules into the host cell cytoplasm. UPEC strains encode a number of virulence factors that are associated with the surface of bacterial cell and are secreted and exported to the site of action [22]. *Salmonella* spp. are important pathogen for humans and animals. They colonize the intestinal tract of humans and farm animals. It can also be present in the intestinal tract of wild birds, reptiles, and occasionally insects.

Feedstuff, soil, bedding, litter, and faecal matter are sources of *Salmonella* contamination in farms [23]. *S. typhimurium* DT104 spread in the 1980s worldwide by human travel and then spread locally by the absence of effective antimicrobials. In fact, this strain commonly carries chromosomally based resistance to five antimicrobials: ACSSuT. *E. coli* and *Salmonella enteric*a as foodborne pathogens, cause the largest number of deaths and has the highest cost burden [24].

Campylobacter jejuni, is also the most prevalent bacterial food-borne pathogen in the industrial world. Chickens are the most important source for human infection. Moreover, the flagellum is not only to facilitate motility but also for secretion of Cia. Another pathogenicity-associated factor is the Cdt A, B, C, important for cell cycle control and induction of host cell apoptosis [25]. All the above-mentioned pathogenic bacteria perform biofilm formation, a mode of growth and survival, in which the bacteria are protected from stressful environmental, conditions. Bacteria grown in biofilms are also known to be 1,000-fold more resistant to disinfectants and antimicrobials [26].

The adhesion test revealed the binding activity of cocoa products towards enteropathogenic bacteria as receptor analogue, so interfering with bacterial pathogenic mechanism.

The adhesion differs among type of product and bacterial isolate as shown in table 4.

Bacteria	Cocoa products' treatment ($V50_s$)										
	BSA	**CREA1**	**CREA2**	**CREA3**	**CREA4**	**CREA5**	**CREA6**	**UNIROMA1**	**CREA7**	**CREA8**	**CREA9**
Escherichia coli EPEC	10.55	7.83	8.01	8.53	8.31	8.01	9.29	9.31	8.8	9.47	7.11
E. coli UPEC	11.99	7.65	7.72	7.84	7.87	7.93	8.07	8.17	8.2	8.8	9.1
Salmonella enteritidis	11.69	10.81	9.54	10.22	9.82	9.00	9.52	9.25	10.07	8.61	9.02
Campylobacter jejuni	11.29	9.42	9.91	10.28	10.85	11.36	11.17	11.71	9.58	10.47	10.33
E. coli ATCC® 1813™	11.71	11.70	11.0	10.85	10.28	9.91	9.42	7.98	7.51	8.65	8.31
S. enteritidis ATCC® 3076™	12.28	12.01	9.31	9.29	8.53	8.31	8.01	7.83	8.8	9.0	10.2
C. jejuni ATCC® 3291™	12.04	11.55	11.78	11.67	10.99	11.45	11.2	11.72	11.88	11.0	11.43
*Lsd	0.3	0.4	0.4	0.5	0.4	0.4	0.5	0.4	0.4	0.5	0.5

Table 4: *Time [h] at which half of the maximal growth yield was reached ($V50_s$) as a measure for adhesion of bacteria. The cocoa products were applied as coating materials in the adhesion test along with BSA as a control. The test products were diluted in PBS buffer to a final concentration of 1% (w/v) and, then, incubated overnight at 4°C. Non-coated wells were included as negative controls in each plate. The OD was determined at a wavelength of 650 nm every 15 minutes during 24 hours. The data represent least squared means. Data followed by different letters within one column are significantly different ($P \leq 0.05$). Products with the lowest $V50_s$ (= the fastest appearance of bacterial growth) bound most bacterial cells.*
**Lsd: Last Significant Difference.*

All cocoa products show binding activity towards the bacterial isolates tested (Table 4, 5). The best activity was against enteropathogenic *E. coli* EPEC and UPEC followed by *C. jejuni* and *S. enteritidis*.

Bacteria	Cocoa products' treatment (Log_{10} count mL^{-1})										
	BSA	**CREA1**	**CREA 2**	**CREA 3**	**CREA4**	**CREA5**	**CREA6**	**UNIROMA1**	**CREA7**	**CREA8**	**CREA9**
Escherichia coli EPEC	2.75	3.78	3.11	2.88	2.66	2.4	1.65	1.5	4.1	4.5	3.58
E. coli UPEC	3.33	4.1	3.92	4.5	5.57	5.29	1.8	2.3	3.9	3.3	5.7
Salmonella enteritidis	1.75	2,3	2.0	2.91	3.42	3.57	3.25	3.42	2.91	3.81	3.56
Campylobacter jejuni	2.16	2.69	2.35	2.09	1.69	1.34	2.2	1.1	2.18	2.0	2.1
E. coli ATCC® 51813™,	3.1	2.99	2.6	4.21	4.56	4.61	4.7	2.33	2.14	2.58	2.64
S. enteritidis ATCC® 3076™	1.48	3.26	2.94	2.36	2.48	2.64	2.85	2.97	2.7	3.21	2.84
C. jejuni ATCC® 33291™	0.47	0.59	1.45	1.68	1.87	1.47	1.41	0.38	1.33	1.98	2.5
Lsd	0.3	0.4	0.4	0.5	0.3	0.4	0.5	0.4	0.4	0.5	0.5

Table 5: *Number of adhering bacteria to the plate coatings. The V50s were also converted to adhering cell numbers of the test bacterium according to Becker., et al. [19,20], using the V50s of the ten-fold dilution series of each tested bacterium. The data represent least squared means. Data followed by different letters within one column are significantly different ($P \leq 0.05$). Products with the highest Log_{10} count mL^{-1} bound most cells of bacteria.*

**Lsd: Last Significant Difference.*

The binding activity of cocoa products could be associated to the presence of melanoidins and phenolic compounds. Melanoidins are produced in Maillard reactions between proteins and sugars during roasting process of cocoa bean. Another important cocoa compounds are proanthocyanidins, also known as condensed tannins, which building blocks are the flavan-3-ols (+)-catechin and (-)-epicatechin by condensation reactions. Flavanols are subjected to progressive polymerization [27,28]. Therefore, as evidenced by HPTLC analysis, depending by post-harvesting processes, catechins are converted first into dimers and progressively until decapolymers [1]. Proanthocyanidins can bind protein and act as receptor analogues towards bacterial adhesins or forming complexes with cell-wall polysaccharides [29]. American cranberry juice (*Vaccinium macrocarpon*) contains high concentrations of anthocyanins, flavonol glycosides, phenolic acids and proanthocyanidins. It is recommended as treatment against infections and prostatitis for its ability to protect the urinary tract from adherence of UPEC [30].

Bacterial adhesins allow the pathogenic bacteria to start contact with host cells, recognize specific receptors host cells surface, colonize specific host tissues and organs, and initiate invasion within the host cells. After these interactions, they cause further events by priming signalling pathways in the host and bacterial cells.

In addition, the adhesins lead bacterial-bacterial interactions and the formation of biofilms [31,32]. If adherence are inhibited then the subsequent infection can also be inhibited. This approach forms the basis of anti-adherence strategies. Cocoa products exert also a biofilm dispersive activity towards enteropathogenic bacteria (Figure 1). The best % of biofilm reduction for *E. coli* EPEC, *E. coli* UPEC, *S. enteritidis* and *C. jejuni* ranges 43 up to 79%, 42 up 69, 42 up to 79, and 43 up to 79 of at 100 µL products' dilution. The CREA 9, a powder cocoa sample, is the most effective against *E. coli* EPEC and *C. Jejuni* (78%), *S. enteritidis* (75%), *E. coli* UPEC (69%). CREA 10, a roasted cocoa beans, follows with, respectively, *E. coli* EPEC and *C. jejuni* (79%), *E. coli* UPEC (68%), *S. enteritidis* (66%), at the same dilution. The

UNIROMA1, a fermented dried cocoa beans sample, shows the lower biofilm dispersive activity in comparison with the other samples, as reported in figure 1. The obtained results let suppose a less content of compounds as anti-biofilm agent as the sample was not a processed product.

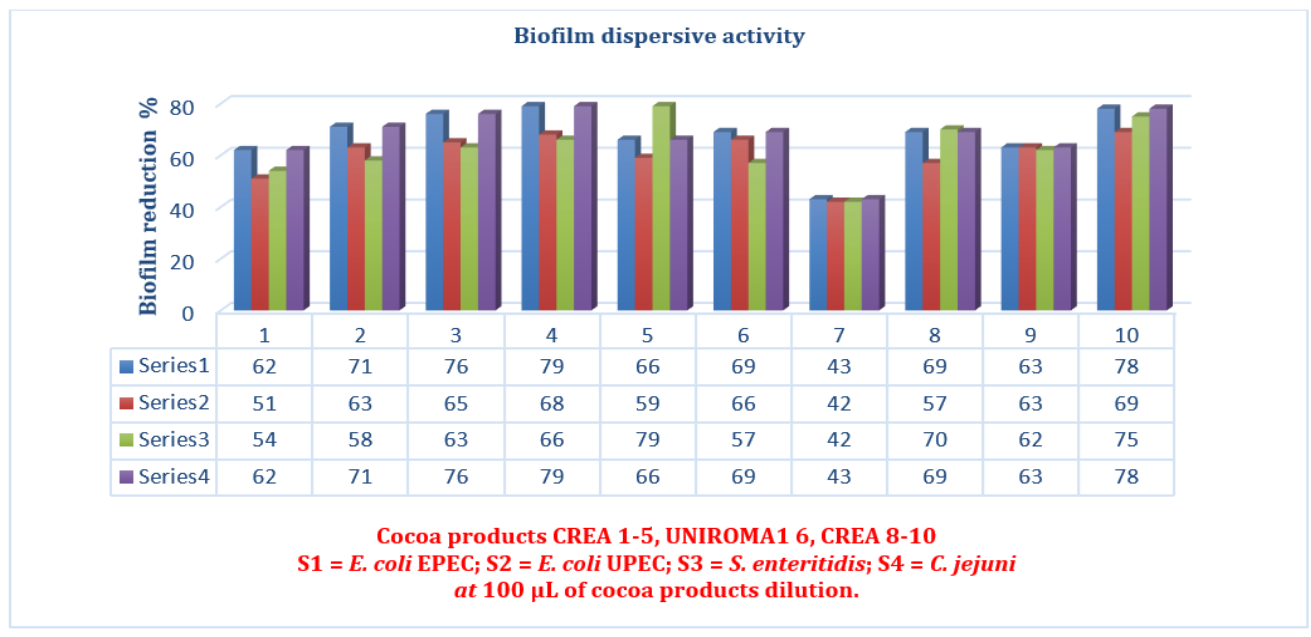

Figure 1: *Biofilm dispersive activity of commercial cocoa products: six roasted cocoa beans (CREA1-CREA6) and three powder cocoa products (CREA7 - CREA9), and one fermented dried cocoa beans, from the UNIROMA1 cocoa sample collection, at 100 µL dilution towards E. coli isolates, S. enteritidis, C. jejuni as biofilm reduction percentage after 24h of incubation at 37°C.*

Until now, many antibiofilm compounds have been identified from diverse natural sources. For example, garlic-derived natural products have been reported to inhibit QS systems in *Pseudomonas* and *Vibrio* species [33]. Phloretin, a flavonoid found in apples controls *E. coli* O157:H7 biofilm formation by inhibiting fimbriae production, necessary for biofilm formation and not having inhibitory activity towards the commensal *E. coli* K-12 biofilm [32]. The cocoa studied products show anti-adhesion activity and anti-biofilm formation towards pathogenic bacteria in different extent, depending by cocoa variety and processing, and tested bacteria [34]. Even though among food melanoidins, those from coffee are by far the most widely investigated, these finding push to strengthen studies for improving analytical techniques to identify the melanoidin structures and to control their formation during thermal cocoa processing. Furthermore, the content and quality of catechins in studied cocoa products especially those coming from Cameron show high catechins content.

Conclusions

The potential health beneficial properties of cocoa as anti-bacterial agent was evidenced. It was related to the type of product and proanthocyanidins, catechins and melanoidins contents. From this starting point, the activity should be, now, validated to allow definition of a recommended daily intake as for other foods [35].

Conflict of Interest

Authors declare no conflict of interest.

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