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Assessment of a garlic product's antibacterial activity and its effects towards enteropathogenic *Escherichia coli*

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Abstract
It is reported a garlic product's antibacterial activity against 48 *Escherichia coli* isolates by standardized disc diffusion agar and micro dilution methods. The average of the Growth Inhibition Zone ranges from 8.13 ± 1.15 to 33.00 ± 1.00. It varies between enteropathogenic and non-enteropathogenic *E. coli* being, respectively, from 21.73 ± 1.35 to 33.00 ± 1.00 and from 8.13 ± 1.15 to 24.33 ± 1.15. It is significantly ($P \leq 0.05$) different with respect to the antibiotic activity that is from 0.00 to 32.75 ± 0.55. The percent growth reduction revealed at 100 µL, 10 µL, 1 µL, and 0.1 µL, garlic product dilutions ranges from 91.51 ± 1.32 to 41. 51 ± 1.15; form 21.79 ± 1.00 to 89.61 ± 0.88; from 11.67 ± 1.33 to 71.19 ± 2.10; and from 11.11 ± 1.08 to 62.28 ± 0.59, respectively. There is a significant difference of antibacterial activity among the isolates and tested product dilutions. The highest percent growth reduction detected at 100 µL concerned mainly enteropathogenic *E. coli* isolates. Viability assays let exclude apoptotic and necrotic effects by garlic product on examined viable *E. coli* populations. Viable cells newly cultured after treatment still grow, but in less extent than the same not treated isolates. The enteropathogenic ones lost virulence factors. This study could represent a possible routinely approach to run quality control of Plant Derived Extract-based products and improve their national and international market in terms of either quality or efficacy. High Performance Thin Layer Chromatography confirmed the extract's composition.

Keywords: Antimicrobials, antibacterial activity; Allium sativum; enteropathogenic Escherichia coli; High Performance Thin Layer Chromatography

1. Introduction
Microorganisms are in almost every habitat in nature and consist of about 60% of the earth's biomass. They are characterized by a noticeable genetic, metabolic and physiological plasticity [1]. Extermination of friend bacteria in favour of resistant ones generates a dangerous disequilibrium in the microbioma. Studies of humans and their microbioma has provided compelling evidence that the composition and activity of resident microbes play crucial roles in shaping the metabolic and regulatory networks that define good health, as well as a spectrum of disease states [2]. Prolonged use of antibiotics led to bacterial adaptation, resulting in the multi-drug resistance in bacteria. After a recent encouraging period of confidence in the antibiotics power, bacteria could become out of control [3, 4].

Escherichia coli is part of the non-pathogenic facultative flora of intestinal tract of humans and other mammals. However, some strains induce diseases of the gastrointestinal and urinary tracts or may affect the central nervous system [5, 6]. There are currently six different groups of pathogenic *E. coli* distinguished based on the mechanism by which they are able to cause disease. Enteropathogenic and Enterohemorrhagic are major causative agents of food poisoning worldwide. All of them cause serious economic losses in farm animal herds and are widespread in newborn in developed and developing countries [7-9]. There is a wide range of transmission possibilities of these pathogens, including direct contact, food, drinks, environment, and others. In fact, they colonize on many types of surfaces, in addition to adhering to different mammalian cells, interacting with other surfaces outside their host, as plants and fruits and distinct overlapping genes family regulates their binding capacity to the different types of surfaces [10]. EPEC and EHEC cause severe intestinal dysfunction, including watery diarrhea or severe bloody diarrhea, and acute kidney failure colonizing and disrupting the linings of intestinal epithelial cells [11]. This is due to interruption of many cellular functions, including the inflammation response that increases the bacteria possibilities of proliferation and survival in the intestine. Upon detection of the pathogenic bacteria presence, epithelial cells and immune cells secrete inflammatory cytokines to enhance the immune response for the efficient removal of the pathogen. Interleukin - 1 beta (Il-1β) is an important inflammatory cytokine for host defenses against enteropathogens. A multimeric protein complex, named the inflammasome, in host cells, regulates its secretion [12]. Enteropathogenic *E. coli* injects a bacterial virulence factor, non-LEE (locus for enterocyte effacement) encoded effector A (Nle-A), to inhibit the secretion of Il-1β. The possible mechanism seems based on the Nle-A interaction directly with Nod-Like Receptor 3 (NLR-3), a cytoplasmic sensor against microbial infection by activating caspase-1 and releasing Il-1β and one of the three basic components of the inflammasome. The presence of Nle-A interrupts the de-ubiquitination of NLR-3, which is a prerequisite for the assembly of the inflammasome. Therefore, Nle-A reduces the formation of the NLR-3 inflammasome and negatively

regulates the secretion of Il-1β [13]. The emergence of antibiotic resistance in pathogenic bacteria has led to a renewed interest in exploring the potential of Plant Derived Antimicrobials (PDA) as an alternative therapeutic strategy to combat microbial infections [14]. This paper deals with a study carried out by a novel metabolomic approach to investigate a whole garlic hydro-alcoholic extract product's antibacterial activity. High Performance Thin Layer Chromatography (HPTLC) was utilized to analyze the chemical composition, in particular for the constituents considered as responsible of the antimicrobial activity. HPTLC, owing to the high automatization and higher separation performance recently obtained, is a useful tool in the analysis of complex mixtures of natural products, such as those introduced nowadays in the market use [15, 16]. Additional biochemical and molecular biology methodologies were used to study the antibacterial activity product's effects against enteropathogenic *E. coli*, too.

2. Materials and methods

2.1 Bacterial Strains and Growth Conditions

A forty-eight isolate collection of *E. coli* isolates was considered. They were different in geographical origin and source of isolation and showed different pathogenetic characteristics. Seventeen were from microorganism's collection of Research centre for fodder and dairy productions of the Council for agricultural research and economics, (FLC isolates), Italy. They were isolated from milk and cheese. All strains were typed both phenotypically and genotypically. Phenotyping was made by the Phene Plate system for *E. coli* (PhP-EC, PhPPlate Microplate Techniques AB, Stockholm, Sweden) and genetic characterization by RAPDNA PCR technique [17]. Seventeen were from microorganism's collection from Central Vietnam Veterinary Institute (CVVI isolates), Vietnam. These microorganisms were isolated from feces of calves affected by diarrhea. Ten were from microorganism's collection of University of Wageningen, (NL isolates), Netherlands. They were isolated from feces of piglets and calves. They are antigenically different and detectable using specific monoclonal antibodies towards different fimbria antigens by *in vitro* agglutination test [18]. Four reference strains were also considered. They were from international culture collections (DSMZ and ATCC) (Table 1). The cultivation/assay medium for *E. coli* was Minca + 1% Iso Vitalex Agar/Broth (Sifin, Berlin, Germany). Bacterial cultures for antibacterial testing were prepared by picking colony from 24-hour-old plates and suspending them in the broth medium (5mL). Cultures grew aerobically for 18 h at 37°C and 100 rpm. For antibacterial activity assay, 1mL of each culture was diluted to 10^5-10^6 CFU/mL. The reference strains grew on media and at the growth conditions as reported on products sheets.

2.2 Garlic extract

A garlic hydro-alcoholic extract marketed as food supplement, Aglio MC™, produced by Caira Laboratories (Villa Latina, FR, Latium, Italy) was used as test starting material (0.33% garlic bulbs). Total composition of the Aglio MC™ was checked by HPTLC. Aglio MC™ was diluted in dimethyl sulfoxide (1 : 1 V/V; Sigma-Aldrich, MI, Italy) under agitation and sterilized by filtration through a 0.22 µm Millipore express filter (Millex-GP,
Bedford, OH, USA) before use.

2.3 HPTLC assay

For each sample, 1 g of raw material was extracted with 5 mL of 70% v/v ethyl alcohol for 24 h, then, the extracts were concentrated to dryness under vacuum. The residues were dissolved in the suitable solvent, in order to perform the analysis (30 mg/ml). There were analyzed fresh clove garlic, aged clove garlic (6 months), denatured clove garlic (it was prepared from fresh garlic by heating a clove in microwave to denature the enzyme), buds of garlic, powdered garlic, dry extract of garlic cloves, hydroalcoholic extract (Aglio MC™). The developed layers were dry at 100°C for 5 minutes, and then derivatized with a selected solution, including NPR (1 g diphenylborinic acid aminoethylester in 200 ml of ethyl acetate). The plate heated at 100°C for 2-3 minutes and then dipped into anisaldehyde-sulfuric acid (1 ml *p*-anisaldehyde, 10 ml H_2SO_4, 20 ml alcohol in 170 ml methanol). Finally, the plates dried for 5 minutes at 120°C before inspection. All treated plates were then inspected under a UV light at 254 or 366 nm or under reflectance and transmission white light, respectively, at a Camag TLC visualizer, before and after derivatisation. WinCATS software 1.4.4 was used for the documentation of derivatized plates. Sample solutions of the extracts resulted stable at 4°C for at least 1 month and for at least 3 days on the plates. Repeatability was determined by running a minimum of three analyses. Retention factor values for main selected compounds varied ± 0.02 %. The effects of small changes in the mobile phase composition, mobile phase volume, duration of saturation were minute and reduced by the direct comparison. On the contrary, the results were critically dependent on prewashing of plates with methanol [19, 20].

2.4 Detection and characterization of the *E. coli* isolates

Molecular biology analysis was used to both detect and characterize *E. coli* isolates and, together with PMA™ dye (Biotium, Hayward, CA, USA), reveals bacteria viable cells after treatment. DNA extraction was performed using Charge Switch® gDNA Mini Bacteria Kit (Life Technologies Italia, Monza, MB, Italy) following manufacturer's instructions. Sixteen primer pairs that specifically amplify *E. coli* 16S rRNA sequences and target genes coding for virulence factors (adhesins and toxins) were considered. The PCR mixtures and conditions are those as reported in the literature. The amplification products' sizes, coordinates, and accession numbers of each primer pair are shown in Table 1 [21-26]. Amplified products (7 µL) were analysed in 2% or 3% agarose gels buffered in 0.5 x TBE buffer (Sigma-Aldrich, Milano, Italy) against a 50 bp, 100 bp, and 1 Kb ladder as size marker (Invitrogen, Milano, Italy). Gel stained with GelRed™ (Biotium, Hayward, CA, USA) in gently agitation at room temperature for ~30 min and visualized by transilluminator (302 nm or 312 nm) (Sigma-Aldrich, Milano, Italy).

Table 1: Primer pairs used to specifically amplify target gene coding for virulence factors (1 – 9: toxins; 10 - 15: fimbriae) of *E. coli* and 16S rRNA sequences

Target gene coding for virulence factors	Oligonucleotide sequences of primers	Amplicon (base pair)	Primer coordinates	Accession number	Reference
1) LT	F 5'ATT TAC GGC GTT ACT ATC CTC3' R 5'TTT TGG TCT CGG TCA GAT ATG3'	281	27-47, 287-307	S60731	[20]
2) Sta	F 5'TCC GTG AAA CAA CAT GAC GG3' R 5'ATA ACA TCC AGC ACA GGC AG3'	244	267-286, 492-510	M58746	[21]
3) STb	F 5'GCC TAT GCA TCT ACA CAA TC3' R 5'TGA GAA ATG GAC AAT GTC CG3'	279	515-534, 773-793	AY028790	[21]
4) Stx1all	F 5'CGC TGA ATG TCA TTC GCT CTG C3' R 5'CGT GGT ATA GCT ACT GTC ACC-3'	302	113-134, 394-414	M17358	[22]
5) Stx2all	F 5'CTT CGG TAT CCT ATT CCC GG3' R 5'CTG CTG TGA CAG TGA CAA AAC GC3'	516	50-69, 543-565	M59432	[22]
6) Stx2e	F 5'ATG AAG AAG ATG TTT ATA GCG3' R 5'TCA GTT AAA CTT CAC CTG GGC3'	264	1176-1196, 1419-1439	M36727	[20]
7) EAST1	F 5'CCA TCA ACA CAG TAT ATC CGA3' R 5'GGT CGC GAG TGA CGG CTT TGT3'	111	2-24, 94-114	S81691	[23]
8) eae	F 5'GGA ACG GCA GAG GTT AAT CTGCAG3' R 5'GGC GCT CAT CAT AGT CTTTC3'	775	1441-1460, 2193-2215	AF022236	[22]
9) hlyA	F 5'AGCTGCAAGTGCGGGTCTG3' R 5'TACGGGTTATGCCTGCAAGTTCAC3'	569	867-885, 1435-1412	X79839	[24]
10) F4 (K88)	F 5'GCT GCA TCT GCT GCA TCT GGTATG G3' R 5'CCA CTG AGT GCT GGTAGT TAC AGC C3'	792	31–54, 798–822	M29374	[25]
11) F5 (K99)	F 5'TGC GAC TAC CAA TGC TTC TG3' R 5'TAT CCA CCA TTA GAC GGA GC3'	450	45-64, 475-494	M35282	[21]
12) F6 (P987)	F 5'TCT GCT CTT AAA GCT ACT GG3' R 5'AAC TCC ACC GTT TGT ATC AG3'	333	193-212, 506-525	M35257	[20]
13) F17	F 5'GGG CTG ACA GAG GAG GTG GGG C3' R 5'CCC GGC GAC AAC TTC ATCACC GG3'	411	289-310, 677-699	AF055313	[25]
14) F18	F 5'GTG AAA AGA CTA GTG TTT ATT TC-3' R 5'CTT GTA AGT AAC CGC GTA AGC3'	510	1-23, 490-510	M61713	[36]
15) F41 F	F 5'GAG GGA CTT TCA TCT TTT AG3' R 5'AGT CCA TTC CAT TTA TAG GC3'	431	154-173, 565-584	X14354	[20]
16) E16SI	F 5'CCCCCTGGACGAAGACTCAC3' R 5'ACCGCTGGCAACAAAGGATA 3'	401	1628-170, 2063–2082	AB035924	[23]
17) E16SII	F 5'AGAGTTTGATGGCTCAG3' R 5'GGACTACCAGGGTATCTAAT3'	798	8-27, 798-805	J01859	[25]

2.5 Assessment of Antibacterial Activity

Disc diffusion method was according to the standard method by Bauer [27]. Bacteria cultures adjusted to 0.5 McFarland standard were smeared on Muller Hinton (MH) II (Becton, Dickinson and Company, Milano, Italy) agar plates using a sterile swab. They were dried for 15 minutes. Three discs impregnated with the extract (100 µL) were placed on the agar surface equidistant to each other. The antibiotic (100 µL wt/v) ciprofloxacin (CFX) (1 mg/mL, Bayer, Milano, Italy) and the dimethyl sulfoxide (DMSO) (VWR International PBI Srl, Milano, Italy, 1mg/mL) were considered as controls. After incubation at 37°C for 18 h, the growth inhibition zone (GIZ) was measured using calipers. The antibacterial activity was also evaluated using microdilution method in conventional sterile polystyrene microplates (Corning, Euroclone SpA, Milan, Italy). Each well was filled with 100 µL of sterile MH II broth (Becton, Dickinson and Company, Milano, Italy) for each bacterial isolate considered; 50 µL of inoculums and amounts of extract at lower dilutions (1 : 10 - 1 : 10,000) were added. Treatment with CFX and sterile distilled water (WTR) were used in the experiment as positive and negative controls. Bacterial growth was determined by OD at 630 nm/10mm path length with an ELISA microplate reader (DynatechML-3000, Pina de Ebro, Spain) after incubation at 37°C for 24 h. Bacterial cell density was transformed to cells/mL using the reference curve equation constructed by diluting at 1 : 100 each bacterial isolate. Counting the number of bacterial cells of an aliquot of this dilution was done using a Neubauer chamber (Celeromics, Vedano al Lambro, MI, Italy). Finally, cell concentrations were transformed to a percentage of bacterial growth reduction (GR). Numbers of viable bacterial cells after treatments were determined by plating aliquots of cell dilutions as CFU/mL, then converted to log CFU and subjected to statistical analyses. Three replicates were considered. The results recorded as means ± SD of the duplicate experiment. Differences between means of data were compared by LSD calculated using the SAS.

2.6 Apoptosis and necrosis detection

The AnnexinV-FITC Apoptosis detection kit (Biotool.com, USA) allows to reveal apoptotic and necrotic effects of garlic product on viable *E. coli* isolates under a fluorescence microscope (Leica Z6APO (A) APOA, Milano, Italy) with excitation: 495 nm - emission: 517 nm and excitation: 538 nm - emission: 619 nm, respectively, according the manufacturer protocol.

2.7 Bioluminescent assay to assess bacteria cellular ATP content

The Bac Titer-Glo™ Microbial Cell Viability Assay (Promega Corporation, Madison, WI, USA) was used for bioluminescent ATP assay according to the manufacturer protocol. Untreated and treated *E. coli* bacterial strains were diluted 50-fold in fresh MH II Broth and then incubated for several hours to reach log phase. Samples of the cultures serially diluted using MH II Broth in a standard opaque-96-well plate. Luminescence recorded using GloMax® Discover System (Promega Corporation, Madison, WI, USA). Signals recorded as the mean of three replicates for each measurement. The signal was calculated as SN = [mean of signal – mean of background]/standard deviation of background] after generating an ATP standard curve according to the protocol (*Glo Max® Discover System Technical Manual* #TM397 and *Bac Titer-Glo™ Microbial Cell Viability Assay Technical Bulletin* #TB337: www.promega.com/protocols/). Bacterial viable cell numbers determined by plate counting of CFU on MH II agar plates.

3. Results

3.1 HPTLC assay

The presence of alliin and carotenoids was achieved by HPTLC analysis. The analysis, reported in Fig. 1, clearly evidences the presence of the two classes of products and is in accordance with fingerprints in literature. In the plate, spots coloured in yellow in the range between Rf 0.6-0.4 values are consistent with compounds related to alliins, consisting in derivatives of autocondensation of intermediates (sulphenic acids). Below this part of the plate, the carotenoids are present as orange spots [28].

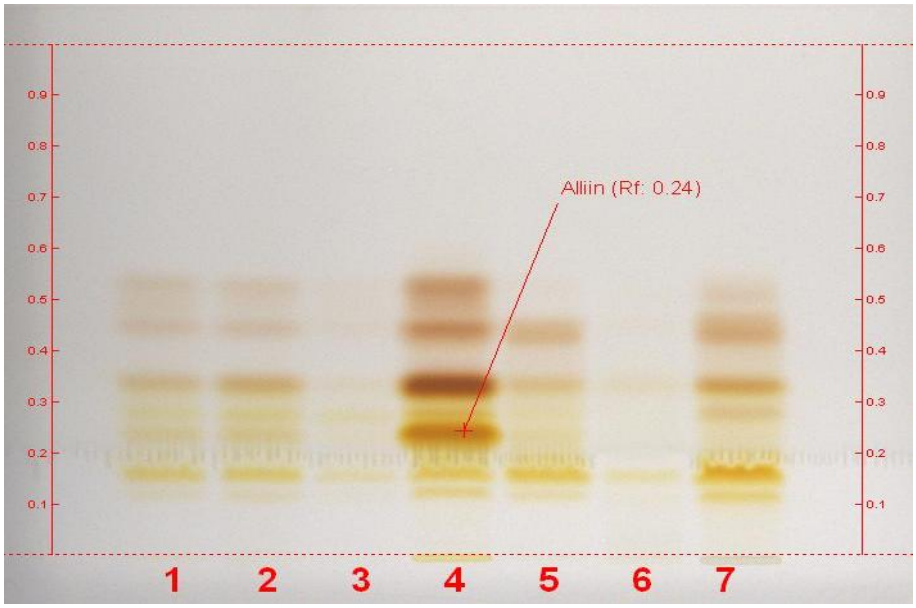

Fig 1: High Performance Thin Layer Chromatography plate. Comparison of different garlic samples. Visualization under white light (WRT) after derivatization in Ninhydrin. Track: 1. Fresh clove garlic; 2. Aged clove garlic; 3. Denatured clove garlic; 4. Buds of garlic; 5. Powdered garlic; 6. Dry extract; 7. Hydro-alcoholic extract (Aglio MC™), **Rf:** Ratio between the migration distance of substance and the migration distance of solvent front

3.2 Detection and characterization of the *E. coli* Isolates

The molecular biology characterization of the forty-eight *E. coli* isolates revealed the presence of fourteen enteropathogenic *E. coli* strains. They were ten *E. coli* isolated from faeces of calves and piglets and four from calves collected, respectively, in Netherlands and Central Vietnam. Their virulence characteristics are in Table 2.

Table 2: Molecular characterisation of enteropathogenic *E. coli* and designation of the strains considered in this study.

E. coli enteropathogenic isolates	Surface antigen	Toxins	Fimbriae
(1) CVVI K10B	nd	STb, LT, EAST1	F4
(2) CVVI KH10	nd	STa, STb	F18
(3) CVVI E12b	nd	STa	F5, F41
(4) CVVI E10	nd	STa	F5, F41
(5) NL K99 /F5	O8K85K99	nr[1]	F5
(6) NL P987	O64:K;9877	STa+	F6
(7) NL K99-1	O8:K25:K99	nr	F5
(8) NL K99-3	O101:K28:K99	nr	F5
(9) NL K99-5	O9:K30:K99	nr	F5
(10) NL K99-7	O101:K32:K99	nr	F5
(11) NL K99-9	O9: K35: K99	nr	F5
(12) NL K99-11	O9:K37:K99	nr	F5
(13) NL K99-15	O20: K?:K99	nr	F5
(14) NL K99-19	O101:K?:K99	nr	F5

[1] not recorded

NL: Department of Bacteriology and Animal Science, University of Wageningen, Netherlands
CVVI: Central Vietnam Veterinary Institute, Vietnam

Table 3: Antibacterial activity of garlic extract against forty-eight *Escherichia coli* isolates revealed as Growth Inhibition Zone.

	Growth Inhibition Zone (mm)*			
***E. coli* isolates**	**Garlic MC™ (100 µL)**	**DMSO (100 µL)**	**WTR (100 µL)**	**CFX (100 µL)**
(1) FLC 1056	9.33 ± 0.58 B	—	—	30.41 ± 0.20 A
(2) FLC 1247	8.13 ± 1.15 B	—	—	30.52 ± 1.07 A
(3) FLC 1059	11.83 ± 1.13 B	—	—	29.62 ± 1.00 A
(4) FLC 1243	19.00 ± 1.00 B	—	—	31.53 ± 0.67 A
(5) FLC 1048	12.33 ± 0.58 B	—	—	29.42 ± 0.58 A
(6) FLC 1167	9.50 ± 0.70 B	—	—	30.61 ± 1.21 A
(7) FLC 1249	13.33 ± 0.58 B	—	—	29.61 ± 1.11 A
(8) FLC 1055	14.53 ± 1.25 B	—	—	31.75 ± 0.82 A
(9) FLC 1054	16.23 ± 1.18 B	—	—	31.41 ± 0.76 A
(10) FLC 1085	18.00 ± 1.00 B	—	—	30.53 ± 1.17 A
(11) FLC 1244	15.33 ± 0.48 B	—	—	28.86 ± 1.00 A
(12) FLC 1165	9.50 ± 0.70 B	—	—	31.33 ± 0.67 A
(13) FLC 1086	11.33 ± 0.58 B	—	—	29.82 ± 0.48 A
(14) FLC 1053	14.53 ± 1.15 B	—	—	32.65 ± 1.39 A
(15) FLC 1095	16.83 ± 1.18 B	—	—	29.05 ± 1.22 A
(16) FLC 1219	10.70 ± 1.00 B	—	—	32.75 ± 0.55 A
(17) FLC 1235	13.23 ± 0.88 B	—	—	30.15 ± 0.55 A
(18) DSM 8696	13.50 ± 0.50 B	—	—	26.21 ± 1.00 A
(19) DSM 9025	13.33 ± 0.58 B	—	—	21.64 ± 0.94 A
(20) DSM 10973	13.53 ± 1.25 B	—	—	29.14 ± 1.75 A
(21) ATCC 33559	13.83 ± 1.18 B	—	—	32.12 ± 1.09 A
(22) CVVI E10	33.00 ± 1.00 B	—	—	20.83 ± 1.65 A
(23) CVVI E173	12.23 ± 0.58 B	—	—	32.35 ± 1.49 A
(24) CVVI E12b	27.50 ± 0.50 B	—	—	11.25 ± 0.68 A
(25) CVVI E16	14.33 ± 0.88 B	—	—	27.54 ± 1.45 A
(26) CVVI E320	21.53 ± 1.35 B	—	—	28.75 ± 1.86 A
(27) CVVI E130	11.83 ± 1.78 B	—	—	29.64 ± 0.87 A
(28) CVVI E48	10.00 ± 1.40 B	—	—	29.31 ± 0.27 A
(29) CVVI KH10	26.33 ± 0.53 B	—	—	15.34 ± 0.66 A
(30) CVVI K10B	27.50 ± 0.56	—	—	0.00
(31) CVVI E298	11.33 ± 0.48 B	—	—	23.57 ± 1.69 A
(32) CVVI E273	13.53 ± 1.75 B	—	—	29.59 ± 1.77 A
(33) CVVI K436	13.14 ± 1.68 B	—	—	30.21 ± 1.38 A
(34) CVVI E98	11.00 ± 1.00 B	—	—	26.91 ± 1.56 A
(35) CVVI E77	14.33 ± 0.58 B	—	—	23.93 ± 0.59 A
(36) CVVI E148	13.50 ± 0.50 B	—	—	28.71 ± 0.87 A
(37) CVVI E10	24.33 ± 0.58 B	—	—	10.35 ± 1.11 A
(38) CVVI E215	16.53 ± 1.15 B	—	—	26.41 ± 1.40 A
(39) NL K99/F5	29.83 ± 1.18 B	—	—	16, 21 ± 0.89 A
(40) NL P987/F5	30.00 ± 1.00 B	—	—	13.21 ± 1.15 A
(41) NL K99-1*	21.73 ± 1.35 B	—	—	15.34 ± 1.37 A
(42) NL K99-3*	28.33 ± 1.5	—	—	0.00
(43) NL K99-5*	25.00 ± 1.10 B	—	—	9.54 ± 1.11 A
(44) NL K99-7*	26.33 ± 0.58 B	—	—	14,25 ± 1.11 A
(45) NL K99-9*	27.50 ± 0.50 B	—	—	11.26 ± 1.78 A
(46) NL K99-11*	29.53 ± 1.25 B	—	—	16.24 ± 1.68 A
(47) NL K99-15*	28.83 ± 1.38 B	—	—	16.35 ± 1.11 A
(48) NL K99-19*	25.00 ± 1.70 B	—	—	15.53 ± 0.84 A

* Three plates for each bacterial isolate; the experiment was performed twice. Results recorded as mean ± SD of the duplicate experiment. Differences between means of data were compared by least significant difference calculated using the Statistical Analysis System (SAS, Institute, Inc. Cary, NC, USA). Values as mean ± SD. Values in a row followed by different lower case letters are significantly different at $P \leq 0.05$.

ATCC: American Type Culture Collection, USA
FLC: Research Centre for Fodder and Dairy Productions of the Council for Agricultural Research and Economics CREA.
CVVI: Central Vietnam Veterinary Institute
DSMZ: Leibniz-Institut, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
NL: Department of Bacteriology and Animal Science, University of Wageningen, Netherlands

3.3 Garlic product antibacterial activity

All the isolates were susceptible to the garlic product dilutions tested even though in different extent. The GIZ (mm) range from 8.13 ± 1.15 to 30.00 ± 1.00. The GIZ varies between enteropathogenic and non-enteropathogenic *E. coli* being, respectively, from 21.75 ± 1.35 to 30.00 ± 1.00 and from 8.13 ± 1.15 to 18.00 ± 1.00. It is significantly ($p \leq 0.05$) different with respect to the antibiotic CFX activity. However, FLC1247 (from milk) resulted to be less susceptible and NLP987/F5 (from piglet's faeces) the most susceptible to Aglio MC™ treatment (100 µL) among all tested bacteria. The CFX GIZ ranges from 0.00 to 32.75 ± 0.55. The enteropathogenic *E. coli* resulted more resistant or less susceptible to CFX than the non-enteropathogenic *E. coli*, showing a GIZ range, respectively, from 0.00 to 26.41 ± 1.40 and from 23.57 ± 1.69 to 32.75 ± 0.55. CVVIK10B (from calve faeces) and NLK99-3* (from calve faeces) both revealed resistance to ciprofloxacin. No GIZ detected in plates treated with negative controls (DMSO and WTR).

The percent GR revealed at 100 µL, 10 µL, 1 µL, and 0.1 µL dilutions range from 31.65 ± 1.15 to 89.15 ± 1.38; from 20.11 ± 0.58 to 89.61 ± 0.88; from 11.67 ± 1.33 to 71.19 ± 2.10; and from 11.11 ± 1.08 to 62.28 ± 0.59, respectively. There is a significant difference of antibacterial activity among the isolates and Aglio MC™ dilutions tested (Table 4). The highest percent GR detected at 100 µL. They concerned mainly enteropathogenic *E. coli* isolates (Table 4).

The PMA™ is a photoreactive dye with high affinity for DNA. It intercalates into DNA forming a covalent linkage upon exposure to intense visible light. It is cell membrane impermeable. When a sample comprising both live and dead bacteria is treated with PMA, only dead cells are susceptible to DNA modification due to their compromised cell membranes [29]. Therefore, selective detection of the sole live cells achieved. It was according to microbiological analysis. Bacterial cell counts showed that the numbers of viable bacterial cells never significantly ($P \leq 0.05$) overcome the inocula concentration used for microdilution test.

Amplicons of the enteropathogenic isolate virulence genes never detected from bacterial viable cells checked in samples treated with 100 µL and 10µL garlic product dilutions. On the contrary, amplicons of the expected size revealed in the same samples using primer pair numbers 16 and 17.

Table 4: Bacterial growth reduction (%) at 24 h in liquid medium with different dilutions of garlic product, using as reference the control treatment (without product).

	Percent growth reduction (%)*			
***E. coli* isolates**	**Garlic MC™ (100 µL)**	**Garlic MC™ (10 µL)**	**Garlic MC™ (1 µL)**	**Garlic MC™ (0.1 µL)**
(1) FLC 1056	49.75 ± 1.33 c	25.61 ± 1.00 b	11.67 ± 1.33 a	11.31 ± 2.08 a
(2) FLC 1247	47.61 ± 1.15 b	44.70 ± 1.00 b	11.80 ± 1.33 a	11.86 ± 1.00 a
(3) FLC 1059	41.61 ± 1.15 c	54.70 ± 1.00 b	14.58 ± 1.33 a	14.86 ± 1.00 a
(4) FLC 1243	58.77 ± 1.10 c	21.79 ± 1.00 b	21.40 ± 0.00 b	16.68 ± 1.20 a
(5) FLC 1048	55.60 ± 1.53 c	43.23 ± 2.08 b	23.39 ± 2.00 b	13.88 ± 1.73 a
(6) FLC 1167	63.71 ± 1.00 c	40.11 ± 0.58 b	21.17 ± 0.00 b	11.18 ± 0.89 a
(7) FLC 1249	59.55 ± 1.53 c	48.61 ± 1.00 b	28.17 ± 1.23 b	18.51 ± 2.08 a
(8) FLC 1055	64.61 ± 1.25 d	48.70 ± 1.00 c	25.18 ± 1.18 b	14.86 ± 1.00 a
(9) FLC 1054	57.31 ± 1.15 d	49.70 ± 1.10 c	26. 18 ± 1.13 b	18.66 ± 1.10 a
(10) FLC 1085	53.50 ± 1.00 c	38.19 ± 1.00 c	28.13 ± 0.10 b	17.54 ± 1.20 a
(11) FLC 1244	75.70 ± 1.53 d	68.73 ± 1.08 c	28.59 ± 2.10 b	12.63 ± 1.23 a
(12) FLC 1165	69.61 ± 1.00 c	59.41 ± 1.58 c	32.27 ± 2.00 b	16.48 ± 1.89 a
(13) FLC 1086	65.75 ± 1.53 c	58.61 ± 1.10 c	28.87 ± 1.23 b	12.41 ± 1.08 a
(14) FLC 1053	64.71 ± 1.15 d	50.70 ± 1.15 c	27.12 ± 1.23 b	16.66 ± 1.00 a
(15) FLC 1095	51.32 ± 1.15 c	29.70 ± 1.21 b	25.00 ± 1.33 a	14.86 ± 1.00 a
(16) FLC 1219	58.85 ± 1.00 d	58.79 ± 1.00 c	28.10 ± 0.00 b	18.68 ± 1.20 a
(17) FLC 1235	66.61 ± 1.53 d	51.81 ± 2.01 c	27.11 ± 1.00 b	16.83 ± 1.13 a
(18) DSM8696	69.34 ± 1.00 d	60.61 ± 1.58 c	25.17 ± 0.00 b	17.38 ± 0.89 a
(19) DSM9025	69.65 ± 1.53 c	55.21 ± 1.17 c	23.67 ± 1.53 b	18.76 ± 2.15 a
(20) DSM10973	54.11 ± 1.15 c	41.28 ± 1.33 c	28.28 ± 1.00 b	18.26 ± 1.00 a
(21) ATCC33559	41.51 ± 1.15 d	49.70 ± 1.23 c	21.78 ± 1.03 b	18.86 ± 1.00 a
(22) CVVI E210	84.90 ± 1.00 c	68.19 ± 1.00 c	69.26 ± 0.00 b	18.38 ± 1.20 a
(23) CVVI E173	76.50 ± 1.33 d	65.13 ± 1.08 c	37.87 ± 2.00 b	22.13 ± 1.13 a
(24) CVVI E12b	89.31 ± 1.01 c	72.00 ± 0.58 c	60.17 ± 1.00 b	48.68 ± 0.89 a
(25) CVVI E16	48.35 ± 1.55 c	31.61 ± 1.00 c	22.57 ± 1.33 b	11.11 ± 1.08 a
(26) CVVI E320	78.51 ± 1.15 d	65.75 ± 1.00 c	27.18 ± 1.33 b	18.81 ± 1.00 a
(27) CVVI E130	80.54 ± 1.15 c	69.73 ± 1.00 c	28.78 ± 1.23 a	14.86 ± 1.00 a
(28) CVVI E48	78.53 ± 1.00 c	68.51 ± 1.00 c	29.33 ± 0.00 a	18.38 ± 1.20 a
(29) CVVI KH10	89.30 ± 1.53 d	71.73 ± 1.08 c	58.39 ± 1.00 b	42.33 ± 1.73 a
(30) CVVI K10B	89.00 ± 1.21 c	89.61 ± 0.88 c	66.37 ± 1.00 b	60.38 ± 0.89 a
(31) CVVI E298	79.15 ± 1.58 c	68.61 ± 1.00 b	28.57 ± 1.33 b	20.51 ± 1.08 a
(32) CVVI E273	64.51 ± 1.15 c	63.70 ± 1.00 c	36.68 ± 1.33 b	21.16 ± 1.00 a
(33) CVVI K436	71.65 ± 1.15 d	59.20 ± 1.00 c	38.10 ± 1.33 b	20.16 ± 1.00 a
(34) CVVI E98	81.93 ± 1.00 d	67.79 ± 1.00 c	41.40 ± 1.00 b	16.68 ± 1.10 a
(35) CVVI E77	84.69 ± 1.53 d	57.73 ± 2.08 b	37.19 ± 2.10 b	19.88 ± 1.63 a
(36) CVVI E148	88.61 ± 1.15 c	53.15 ± 1.28 c	37.17 ± 0.64 b	20.25 ± 1.69 a

(37) CVVI E10	89.15 ± 1.38 c	77.11 ± 1.10 c	60.61 ± 1.13 b	51.19 ± 1.10 a
(38) CVVI E215	64.11 ± 1.15 c	44.74 ± 1.10 c	29.58 ± 1.33 b	24.38 ± 1.00 a
(39) NLK99/F5	91.11 ± 1.15 d	84.21 ± 1.20 c	64.58 ± 1.16 b	60.26 ± 1.22 a
(40) NLP987/F5	89.19 ± 1.00 c	88.19 ± 1.10 c	61.10 ± 0.10 b	53.18 ± 1.18 a
(41) NLK99-1∗	91.51 ± 1.32 d	81.53 ± 1.05 c	71.19 ± 2.10 b	50.33 ± 1.63 a
(42) NLK99-3∗	89.11 ± 1.00 c	78.41 ± 0.48 c	67.15 ± 0.10 b	51.28 ± 1.39 a
(43) NLK99-5∗	89.15 ± 1.53 c	71.21 ± 1.10 c	61.11 ± 1.33 b	50.51 ± 2.18 a
(44) NLK99-7∗	86.26 ± 1.15 c	79.72 ± 1.00 c	65.48 ± 1.33 b	54.66 ± 1.00 a
(45) NLK99-9∗	91.41 ± 1.25 c	86.11 ± 1.00 c	67.28 ± 0.13 b	60.21 ± 1.00 a
(46) NLK99-11∗	87.90 ± 1.10 c	78.19 ± 1.00 c	67.45 ± 0.00 b	61.38 ± 1.12 a
(47) NLK99-15∗	91.33 ± 1.23 d	88.13 ± 1.18 c	62.33 ± 1.28 b	54.60 ± 1.73 a
(48) NLK99-19∗	89.31 ± 1.10c	81.11 ± 0.78 c	70.21 ± 0.00 b	62.28 ± 0.59 a

3.4 Viability assays after E. coli isolates treatment

Observation at the fluorescence microscope never revealed green and/or red fluorescence. This let exclude apoptotic and necrotic (green fluorescence) bacterial cells presence. In fact, Annexin V conjugated to FITC mark the phosphatidylserine sites on membrane apoptotic cells after releasing of phosphatidylserine, a phospholipid membrane component that plays a key role in cell cycle signalling, specifically, in relationship to apoptosis. In addition, PI, also included in the kit, being membrane impermeable reveals dead cells counterstaining in multicolour fluorescent technique (red fluorescence). The bioluminescent assay is a responsive, rapid, and relatively inexpensive system, useful for the large-scale screening of samples in a one-day work [30]. It is based on the measurement of ATP, a universal energy unit in all living cells to determine the number of metabolically active cells. The luminescent signal is proportional to the amount of ATP present, which is directly proportional to the number of viable cells in culture. The bioluminescent assay was in agreement with the other tests considered. It showed that after treatment viable enteropathogenic *E. coli* isolates cells newly cultured still grow. Their growth resulted significantly lower than growth of the same not treated isolates (Table 5).

Table 5: *E. coli* CVVI K10B, NLK99-15 and NLK99-3 isolates bacterial cell numbers and luminescent signal after treatment with garlic product grew in MH II Broth at 27°C up to 6 h.

Time (h)	Parameter	Initial titer of *E. coli* suspension (CFU/mL)					
		CVVI K10B		NLP987/F5		NLK99-3*	
		Untreated	Treated	Untreated	Treated	Untreated	Treated
3	CFU/mL	$1.04 \pm 0.23 \times 10^7$ a	$1.41 \pm 0.23 \times 10^4$b	$1.46 \pm 0.36 \times 10^6$a	$1.05 \pm 0.11 \times 10^4$b	$1,22 \pm 0.27 \times 10^6$ a	$1.55 \pm 1.12 \times 10^4$b
	ATP mol/mL	17.42 ± 3.56 a*	7.22 ± 0.56 b	21.22 ± 1.36 a	6.72 ± 1.32 b	$18.14 \pm 0.84 \times 10^6$a	8.71 ± 1.34 b
4	CFU/mL	$2.64 \pm 0.56 \times 10^8$ a	$2.31 \pm 0.23 \times 10^4$b	$1.88 \pm 0.36 \times 10^7$ a	$1,71 \pm 0.36 \times 10^4$ b	$1,03 \pm 0.27 \times 10^7$ a	$2.45 \pm 1.12 \times 10^5$b
	ATP mol/mL	24.56 ± 4.34 a	10.38 ± 1.14 b	28.11 ± 3.24 a	7.26 ± 1.38 b	20.20 ± 2.24 a	14.16 ± 1.38 b
5	CFU/mL	$1.64 \pm 0.21 \times 10^9$ a	$2.24 \pm 0.23 \times 10^4$b	$2.06 \pm 0.36 \times 10^8$ a	$2,11 \pm 0.66 \times 10^4$ b	$1,91 \pm 0.27 \times 10^8$	$8.45 \pm 1.12 \times 10^5$b
	ATP mol/mL	28.42 ± 2.34 a	18.61 ± 1.34 b	31.22 ± 1.44 a	12.61 ± 1.64 b	29.22 ± 2.34 a	22.31 ± 2.64 b
6	CFU/mL	$2.27 \pm 0.38 \times 10^9$ a	$3.24 \pm 0.23 \times 10^4$b	$3.21 \pm 1.26 \times 10^9$ a	$1,71 \pm 0.26 \times 10^5$ b	$3,73 \pm 0.27 \times 10^9$ a	$1.15 \pm 1.12 \times 10^6$b
	ATP mol/mL	31.45 ± 3.12 a	21.13 ± 3.12 b	35.18 ± 2.11 a	18.15 ± 2.12 b	37.22 ± 2.34 a	25.81 ± 2.64 b

*Values are given as mean ± Standard Deviation Values in a row followed by different lower case letters are significantly different at $p \leq 0.05$.

4. Discussion

The data here reported let us suppose that garlic product antibacterial activity acts on adhesion factor, membrane, and its permeability with possible loss of extrachromosomal DNA. Scientific evidences show bacteria can avoid the effects of antibiotics when being in a physiological state in which the antibiotics do not kill them, knew as persistence [31, 32]. A ubiquitous bacterial stress alarmone is the central regulator of multi-drug tolerance and persistence. While bacterial persistence is non-inherited, the propensity to form persister cells is nevertheless a genetically evolved trait. [33]. If persister cells are naturally occurring presence among bacterial populations with unequal behaviour of individuals or not is still an open question. They grow more slowly than normal cells under unstressed growth conditions, but survive longer under stress conditions such as the treatment with antibiotics [34]. The human action increases hugely the pressure on microbial communities. The rate at which a microbial community returns to its original composition after being disturbed exponentially increase, too. On the contrary, it seems that human being have lost this natural capability. This has significantly limited the efficacy of antibiotics, requiring alternative strategies to strive microbial infections. The EFSA and ECDC report AMR poses a serious risk to human and animal health, with bacteria in humans, food and animals continuing to show resistance to such drugs. AMR to widely used antibiotics, such as CFX, is commonly detected in bacteria in poultry, broiler and turkey meat. AMR to CFX is very high in *Campylobacter* spp, thus reducing the possibilities for effective treatment of severe foodborne infections [35]. Garlic seems an ideal antimicrobial agent being effective against both on pathogens and host immune response. The antimicrobial activity of garlic extract correlates with the allicin content [36]. Allicin is active against human pathogens that are resistant against certain antibiotics. A prominent case is represented by methicillin resistant *Staphylococcus aureus* (MRSA) the main cause in many hospital infections. However, the antibacterial role of alliin, allicin and other sulfur-rich (cicloalliin, isoalliin e metiin) antibacterial compounds in garlic extracts it is not yet clarified since its instability in the presence of other garlic compounds. In addition, allicin has been reported for its immunomodulatory, anti-inflammatory, and antitumor [37-39], activities. It modulates immune responses, enhances natural killer activity and T-lymphocyte proliferation and thus affect growth performance most positively. The several biological activities of garlic compounds support the importance to study

and use the plant whole phytocomplex or its fractions instead of its single substances so as phytocomplex synergistically act on host/pathogen (or parasite) system containing disease expression.

5. Conclusions and future Implications

Garlic products are marketed in the EU as either foodstuffs or herbal medicinal products. The first are the most on the European market and they are often sold with health claims. On the contrary, garlic drugs are available only in eleven of the twenty-eight EU member states through a national authorization procedure. This is due mainly because of producers meet many difficulties to accomplish all the requirements of European directives and guidelines for medicinal products.

This paper reports the antibacterial activity of a garlic food supplement against *E. coli* adapting the validation to the particular type of botanical product. Based on its antibacterial activity, it should have several field relating human and animal health and wellbeing as well as feed and food preservation. This study could represent a routinely approach for the quality control of Plant-Derived –Extract-based product and improve their national and international market in terms either of quality or efficacy.

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