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Evaluation of the Biological Activities of a Multicomponent-Product as Sheep and Goat Feed Supplement

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Abstract

The *in vitro* assessment of the biological activities of a multicomponent-product as sheep and goat feed supplement towards enteropathogenic bacteria as well as inflammation-associated-bacteria is reported. The bacteria, namely, *Clostridium perfrigens, Escherichia coli, Staphylococcus aureus, Streptococcus uberis*, and *Pseudomonas aeruginosa* were considered in the experiment. The percent bacterial growth reduction revealed at 100 µL, 10 µL, 1 µL, and 0.1 µL product dilutions was in the range 50.81 ± 1.15 - 86.31 ± 1.15; 37.21 ± 2.11 - 81.20 ± 1.38; 5.23 ± 1.69 - 67.56 ± 1.13; and 3.60 ± 0.89 - 51.88 ± 1.00, respectively. There is a significant difference of antibacterial activity among the isolates and the dilutions tested. In addition, the product showed adhesive activity and biofilm dispersive activity. The highest binding activities were recorded at 100 µL and 10 µL dilutions towards: *C. perfrigens* (V_{50} 5.8), *E. coli* NLK99-3 (V_{50} 5.6), *E. coli* CVVI KH10B strain (V_{50} 5.2), *S. aureus* (V_{50} 6.3), *P. fluorescens* (V_{50} 5.6), and *E. coli* ATCC® 51813™ (V_{50} 7.0), *S. aureus* ATCC® 29213™ (V_{50} 6.8), *Str. Uberis* ATCC® 700407™ (V_{50} 6.7), and *P. aeruginosa* (V_{50} 6.3), respectively. The highest biofilm dispersive activities were recorded using 100 µL and 10 µL of product dilutions towards *S. uberis* (4.5%), *E. coli* CVVI KH10B (4.5%), *P. aeruginosa* (4.31%), *E. coli* NLK99-3 (4.3%), and *E. coli* CVVI KH10B (4.4%), *C. perfrigens* (3.31%), *P. aeruginosa* (3.1%), respectively. Finally, the product anti-inflammatory activity as inhibitor of COX enzymatic activity was investigated. The highest percent inhibitions were observed at 10 µL (COX1 33.2 ± 1.4; COX2 33.2 ± 1.4) and 1 µL (COX1 39.2 ± 1.4; COX2 32.1 ± 0.9) product treatments. Molecular biology analysis let to either identify and characterize isolated microorganisms or together with PMA™ dye to detect bacteria viable cells after product treatments. The applied methodologies let to reveal the efficacy of the feed additive to constrain, as claimed in product label, microorganisms attack and inflammatory processes that can alter the herd wellbeing and the quality of animal production.

Keywords: *Adhesion Activity; Biofilm Dispersive Activity; Anti-Inflammatory Activity; Animal Nutrition; Enteropathogenic Bacteria; Antimicrobial Resistance*

Abbreviations

ATCC: American Type Culture Collection; BHI: Brain Heart Infusion agar/broth; BPA: Baird Parker agar/broth; BSA: Bovine Serum Albumine; CBAB: Columbia Blood Agar Base/broth; DBH: Defibrinated Horse Blood; DHAB: Defibrinated Horse Blood Agar; DMSO: Dimethyl Sulfoxide, DSB: Defibrinated Sheep Blood; EDTA = Ethylenediaminetetraacetic Acid; GRBC: Goat Red Blood Cells; MBCE: Minimum Biofilm Eradication Concentration; MSA: Mannitol Salt Agar/broth; RBC: Red Blood Cells; PIA: Pseudomonas Isolation Agar; SD: Standard deviation; SSS: *Streptococcus* Selective Supplement; TAE: Tris acetate-electrophoresis buffer; PBS = Phosphate-buffered saline; WBC: White Blood Cells; TMPD: N, N, N, N'-tetramethyl-pphenylenediamine; TSA = Trypticase Soy Agar/broth

Introduction

After the prohibition on the use of antibiotics as in-feed growth promoters by the European Community in 2006, an increase in the occurrence of post-weaning diarrhea in pigs, calves and lambs, necrotic enteritis associated with *Clostridium perfringens* in broilers, mastitis, laminitis, as well as in the therapeutic use of antibiotics was nodded [1]. Infection with *C. perfringens* types B and C causes severe enteritis, dysentery, toxemia, and high mortality in young lambs, calves, pigs. Types B and C both produce the highly necrotizing and lethal beta toxin responsible for severe intestinal damage. Type A also causes enterotoxaemia in adult cattle, sheep, and goats [2]. The potential hazard of *C. perfrigens* heat-resistant spores is great in those areas where they frequently occur www.infectiousdisease.dhh.louisiana.gov.

Escherichia coli is another bacterium associated to diarrhea during weaning and post-weaning. Enteropathogenic strains of *E. coli* adhere to the intestine and produce so-called attaching and effacing lesions, with dissolution of the brush border and loss of microvillus structure at the site of attachment, a decrease in enzyme activity, and changes in ion transport in the intestine. Some strains produce toxin causing severe hemorrhagic diarrhea [3].

Mastitis is the inflammation of the mammary gland and udder tissue, and is a major endemic disease of dairy herds. A high prevalence of mammary infection contribute to an increase in total bacteria in milk, which is a legally restricted parameter in milk production in the European Union (DIR 94/71/CE, 1994, DIR 98/8/CE,).

Bacteria associated with the goat syndrome are: *Staphylococcus aureus, Arcanobacterium pyogenes, Bacillus* spp., *Clostridium perfringens, Nocardia asteroids* [4,5].

Risks for the consumer derive from the pathogens presence in the milk, which will avoid if the proper heat treatment is applied, and by the toxins presence. In fact, staphylococcal enterotoxins are thermostable and persist in dairy products processed with milk from infected animals despite being pasteurized or sterilized.

Laminitis in goats spread out worldwide, but the incidence is lower than that in dairy cattle and horses. Often this syndrome convoluted by systemic bacterial infection. The main associated bacteria are *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp. [6].

Bacteria colonize on many types of surfaces. The adherent state is advantageous for bacterial survival and a key step in pathogenesis. Adhesion is one of the initial stages of the infectious process. Adhesion of enteric, oral and respiratory bacteria is required for colonization and for subsequent development of disease [7].

Biofilm formation represent another key role for environmental survival but also for successful infections by various pathogenic bacteria. The biofilm formation include initial attachment, colony formation and construction of biofilm by secretion of extracellular polymeric substances. Bacterial biofilms are resistant to antimicrobials and disinfectants. Biofilm formation has also been implicated in a wide range of human bacterial persistent infections [8].

All the above-mentioned diseases imply considerable economic losses owing to increased morbidity and mortality, decreased growth rates and cost of medication [9]. In addition, food-borne zoonotic diseases are a significant and wide spread public health threat. Indeed, animal product are source of zoonotic enteropathogenic bacteria even though from asymptomatic animal. In fact, enteropathogenic bacteria can also contaminate muscle after culling via diapedesis.

Nowadays alternatives for in-feed antibiotics need especially because of the increase of antimicrobial resistance. Phytocomplex represents an innovative alternative to antibiotics as remedies or feed supplements in zootecnic [10].

This paper deals with the assessment and evaluation of the biological activities of a multicomponent product used as feed supplements for sheep and goats that claims antibacterial and anti-inflammatory activities.

Materials and Methods

Multicomponent feed additive

ALGATAN™, a commercial feed additive for sheep and goat, by Lombarda Trading (CasalbuttanoedUniti, CR, Italy), was used as test starting material. The product was diluted in sterilized distilled water and Tween 80 (1 : 1 V/V; VWR, PBI International, MI, Italy) under agitation and sterilized by filtration through a 0.22 µm Millipore express filter (Millex-GP, Bedford, OH, USA) before use in the experiment.

Bacterial strains and growth conditions

The bacteria, namely, *Clostridium perfrigens*, *Escherichia coli, Staphylococcus aureus, Streptococcus uberis* spp. and *Pseudomonas aeruginosa* were considered in the experiment. The tested *E. coli* strains were collected from calve, pig feces, and previously characterized [11]. *St. aureus, Str. uberis* spp. and *P. aeruginosa* came by smear swabs isolation on udders of dairy goat with symptoms of mastitis as follow.

Bacteria isolation made by a non-invasive technique for microbiological sampling performing the removal using flocked swabs (FLOQ Swabs™ patented technology, Copan, Brescia, Italy). Flocked swabs aimed to collect more sample volume than traditional swabs and automatically and completely release the sample immediately when in contact with liquid or surface. ESwab and two selective enrichment broths for single organism screening: TSB salt broth and LIM broth (Copan, Brescia, Italy) used respectively for *S. aureus* and *Streptococcus* spp. The isolation from ESwab, TSB salt and LIM broth are compliant with the Quality Control of Microbiological Transport Systems; Approved Standard, M40A2 (CLSI M40-A2, 2014) [12] as per guideline DIN 58942-4 Supplement 1 (DFNV, 2004) [13] and performed according to the manufacturer's instructions. The swab method was applied by taking three smears per sample on an area of 10 cm^2 defined with a delimiter. Then, adding 100 µL of isotonic eluent furnished by the Manufacturer for decimal dilutions and plating 1 mL per dilution on selected media using a sterile bacteriology loop to streak the primary inoculum across the surface of the second, third and fourth quadrants of the agar culture plate.

Coliforms with *E. coli* distinction were counted on Chromogenic Compact Dry and Chromogenic Compact Dry with *Escherichia coli* distinction (PBI, Milano, Italy) after incubation at 35°C for 20 hours [14,15]. Then the cultivation/assay medium for enteropathogenic *E. coli* was Minca + 1% IsoVitalex Agar/Broth (Sifin, Berlin, Germany).

BPA (Acumedia, Neogen, lansing MI, USA) was used as selective medium for the isolation and presumptive identification of coagulase-positive *staphylococci*. It contains 7.5% sodium chloride and thus selects for those bacteria, which can tolerate high salt concentration.

MSA is both a selective and differential medium used in the isolation and distinction of *S. aureus* and *S. epidermidis*. Coagulase-negative *Enterococcus* spp. and *Streptococcus* spp. were isolated on CBAB, SSS and DHB (OXOID, Basingstoke, UK) incubated plates at 37°C, either aerobically or anaerobically and examined after 18 to 24 hours incubation. PIA (Acumedia, Neogen Corporation, lansing, MI, USA) was used for the isolation of *Pseudomonas* spp. under aerobic incubation at 35 ± 2°C and examined for growth after 18 - 48 hours. The medium includes Irgasan, a broad-spectrum antibiotic not active towards *Pseudomonas* spp. This medium is selective and formulated to enhance formation of blue or blue-green pyocyanin pigment by *P. aeruginosa*. The pigment diffuses into the medium surrounding growth (Table 1).

An isolate of *C. perfrigens* from ATCC (*C. perfringens* ATCC® 13124™) was also considered in the experiment.

Bacteria	Growth media and conditions	CFU/mL
Clostridium perfringens	TSA + DSB 37°C anaerobic (80% N2 10% CO_2 10% H2)	1.4 x 10^4
Coliform	CCD 35°C 20 h	5.3 x 10^7
E. coli	CCD EC 35°C 20 h	5 x 10^5
E. coli (CVVI KH10 and NLK99-3 isolates)	Minca + 1% IsoVitalex Agar/Broth 35°C 20 h	5 x 10^6
Coagulase positive	DBPA	6.4 x 10^4
S. aureus	TSB	3.8 x 10^6
	MSA	2.3 x 10^7
Coagulase negative	CBAB 37°C aerobic and anaerobic 18 to 24 h	1.3 x 10^5
Streptococcus group	LIM	2.3 x 10^5
Streptococcus spp.	CBAB + SSS 37°C 18 to 24 h	1.8 x 10^4
Enterococcus spp.	CBAB + DHB 37°C 18 to 24 h	2.8 x 10^6
Pseudomonas spp.	PIA 35°C 18 to 48 h	1.6 x 10^5

Table 1: *Synopsis of bacteria growth media, conditions, and enumerations.*

E. coli, American Type Culture Collection (ATCC)® 51813™, *S. aureus* ATCC® 29213™, E. faecalis ATCC® 7088™, *S. uberis* ATCC® 700407™, and *P. aeruginosa* ATCC® 27853™ were used as reference strains. The reference strains grown on media and at the growth conditions as reported on products sheets.

To prepare working cultures, stock cultures standardized through two successive 24h growth cycles in BHI broth (Difco, Buccinasco, MI, Italy) at 20°C without agitation.

Identification and characterization of bacterial isolates

Molecular biology analysis was used to either identify and characterize isolated microorganisms or together with PMA™ dye (Biotium, Hayward, CA, USA) to detect bacteria viable cells after ALGATAN™ treatment *in vitro*. DNA extraction performed using Charge Switch® gDNA Mini Bacteria Kit (Life Technologies Italia, Monza, MB, Italy) following manufacturer's instructions. The molecular identification and characterization made by PCR using specific oligonucleotide primer pairs designed according to species-specific parts of the 16S rRNA gene, and virulence genes for each isolated bacterium as mentioned in the section microbiological analysis. Mixture and reaction conditions as reported in literature (Table 2). Amplified products were determined by electrophoresis of 12 µl of the reaction product in a 2% agarose gel with TAE (40 mmol of Tris-HCl per liter, 1.14 mol of glacial acetic acid per liter, 1 mMol of EDTA per liter [pH 8.0]) with a 100-bp and 1Kb DNA ladder (Gibco BRL, Eggenstein, Germany) as molecular size markers.

Bacteria	Target gene	Primers oligonucleotide sequenze	Amplicon size	Accession number	Reference
Clostridium perfringens ATCC® 13124™	Cpa	F 5'-GCTAATGTTACTGCCGTTGA -3' R 5'-CCTCTGATACATCGTGTAAG-3'	324	nr	[17]
Escherichia coli	E16SI	F 5'-CCCCCTGGACGAAGACTCAC-3' R 5'-ACCGCTGGCAACAAAGGATA -3'	401	AB035924	[18]
E. coli NLK99-3	F5 (K99)	F 5'-TGC GAC TAC CAA TGC TTC TG-3' R 5'-TAT CCA CCA TTA GAC GGA GC-3'	450	M35282	[19]
E. coli CVVI KH10B	F4 (K88)	F 5'-GCT GCA TCT GCT GCA TCT GGTATG G-' R 5'-CCA CTG AGT GCT GGTAGT TAC AGC C-3'	792	M29374	[20]
	EAST1	F 5'-CCA TCA ACA CAG TAT ATC CGA-3' R 5'-GGT CGC GAG TGA CGG CTT TGT-3'	111	S81691	[21]
	Stb	F 5'-GCC TAT GCA TCT ACA CAA TC-3' R 5'-TGA GAA ATG GAC AAT GTC CG-3'	279	AY028790	[22]
	LT	F 5'-ATT TAC GGC GTT ACT ATC CTC-3' R 5'-TTT TGG TCT CGG TCA GAT ATG-3'	281	S60731	[23]
Staphylococcus aureus	Nunc 1	f 5' - ATG AAG TCA AAT AAA TCG CT- 3' r 5'- TTT GGT GAA AAA TAC TTC TC - 5'	458	NC_002745	[24]
Streptococcus uberis	pauA	GSub5'-S TGA TTC CGA CTA CTA CGC TAG AT-3' GSub5'-AS ATA CTT TGA GTT TCA CCG AGT TC-3'	723	FJ196527	[25]
Pseudomonas aeruginosa	O-aag	PA431CF 5'-CTGGGTCGAAAGGTGGTTGTTATC-3' PA431CR 5'-GCGGCTGGTGCGGCTGAGTC-3'	232	NP_253925	[26]

Table 2: *Species-specific primer pairs for bacterial detection using PCR at reaction conditions and mixtures as reported in literature. Cpa: gene expressing alpha toxin; STb: heat-stable enterotoxins; LT: heat labile exotoxin; EAST 1: enteroaggregative heat-stable enterotoxin 1; F4, F5 types of E. coli fimbriae; Nunc I extracellular thermostable nuclease (thermonuclease [TNase]); PauA: plasminogen activator A (PauA) virulent factor; O-aag; O-antigen acetylase gene.*

Biological activity assay

Microdilution assay

Antibacterial activity was assayed by microdilution method using conventional sterile polystyrene microplates of 96 wells (Corning, EurocloneSpA, Milan, Italy); each well was filled with 100 µL of sterile suitable liquid media for each microorganism considered, 50 µL of inoculums and the amounts of product at dilutions 1:10 up to 1:10,000. Control treatment without product used in the experiment. The microplates incubated at 37°C for 24h. Bacterial growth was determined by OD reading at 630 nm/10 mm path length with an ELISA microplate reader (DynatechML-3000, Pina de Ebro, Spain).

Bacterial cell concentration transformed to cells/mL using the reference curve equation. The reference curve was constructed by diluting at 1 : 100 each bacterial isolate.

Counting the number of bacterial cells of an aliquot of this dilution done using a Neubauer chamber (Celeromics, Vedano al Lambro, MI, Italy). Finally, cell concentrations transformed to a percentage of bacterial inhibition. The percentage of bacterial growth reduction (GR %) was estimated using as reference the control treatment (*T*= without extract):

$$Growth\ Reduction\ Percent = (C - T/C)\ X\ 100$$

Three replicates considered. The results recorded as mean ± S.D. of the duplicate experiment. Differences between means of data compared by least significant difference (LSD) calculated using the SAS.

Adhesion test

The adhesive capacity of ALGATAN™ against enteropathogenic *E. coli* isolates tested according to Becker and Galletti [27].

The bacteria suspended in PBS, were added into the microplate wells (300 µl/well) and allowed to adhere at room temperature for 30 min. The wells were afterwards washed three times with 300 µl of PBS to remove non-adherent bacteria. Then the wells were filled with 300 µl growth medium.

The control wells filled with 300 µl of a ten-fold dilution series in growth medium with a known amount of the test bacterium. Then, the microplate was placed in a microplate reader (Thermo Scientific Multiskan GO, Thermo Fisher Scientific, Vantaa, Finland), 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 7; 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, $V_{50}(t)$ is the time at which half of the maximal yield has been reached. Analyses of variance performed using SAS analysis. The V_{50}s also converted to adhering cell numbers of the test bacterium using the V_{50}s of the ten-fold dilution series of the test bacterium [28].

Biofilm dispersion assay

The microorganisms in a microtiter plate are in a form of suspension, whereas the bacteria associated with different illnesses form biofilms. Biofilms are notoriously difficult to eradicate and are a source of many recalcitrant infections, thus this represents an extra challenge for antimicrobial agents [29, 30]. For this reason, the biofilm dispersive activity of the product also checked.

The model for biofilm formation carried out using the MBEC™-HTP from Innovotech, Inc (Edmonton, Alberta, Canada). 200 µL of a 2×10^5 bacteria/ml were added to the MBEC™-HTP. The plates covered with a lid containing pegs for the attachment of the bacteria, and incubated at 37°C per 48h over a rocking table at 12 cycles/min. The assay involves the formation of 96 identical biofilms on plastic pegs on the lid of the MBEC Device for MBEC™ Assay.

These biofilms are then exposed to test different product dilutions (100 µL of 1:10 up to 1:10,000) for a defined time period, then placed in fresh medium for each bacterium in a second 96-well plate (Corning®, Amsterdam, The Netherlands) and incubated overnight. The assay performed according to the Manufacturer's procedure [31].

The Log_{10} density per mm^2 for each peg calculated as:

$$Log_{10}(CFU/mm^2) = Log_{10}[(X/B)\ (V/A)\ (D)+1]$$

where: X = CFU counted on spot plate; B = volume plated (200 µL); V = well volume (200 µL); A = peg surface area (46,63 mm^2); D = Dilution

While, the Log_{10} reduction of each dilution calculated as follow:

$$Log_{10} \text{ reduction} = \text{Mean } Log_{10} \text{ reduction Control pegs - Mean } Log_{10} \text{ treated pegs.}$$

COX inhibition assay

The anti-inflammatory activity of ALGATAN™ tested towards COX1 and COX2. COX1 constitutively expressed while COX2 expressed under different stimuli and is a target enzyme for the anti-inflammatory activity of non-steroidal anti-inflammatory drugs.

The assay was carried out by Colorimetric COX inhibitor Screening assay kit (Cayman Chemical, MI, USA) in a microtiter plate with 96 well for colorimetric assay (Corning®, EurocloneSpA, Milan, Italy).

According to the manufacturer's protocol [32], the reaction mixture contains, 150 ml of assay buffer, 10 ml of heme, 10 ml of enzyme (either ovine COX-1 or COX-2), and 10 µl of ALGATAN™ (1 mg/ml in DMSO).

The assay utilizes the peroxidase component of the COX catalytic domain.

The peroxidase activity was assayed colorimetrically at 590 nm by monitoring the appearance of oxidized TMPD in a microplate reader (Thermo Scientific Multiskan GO, Thermo Fisher Scientific, Vantaa, Finland). Aspirin (acetylsalicylic acid 1 mg/1mL) used as a standard drug. The percent COX inhibition was calculated using following equation:

$$\text{COX inhibition activity \%} = 1 - (T/C) \times 100$$

Where T = Absorbance of the inhibitor well at 590 nm.
C = Absorbance of the 100 % initial activity without inhibitor well at 590 nm.

Results and Discussion

Microdilution assay

The percent bacterial growth reductions, determined in liquid medium with or without ALGATAN™, and reported in Table 3.

The results show that ALGATAN™ has a broad spectrum of antibacterial activity against the tested bacteria even though in different extent. It is dose dependent.

The percent bacterial growth reduction revealed at 100 µL, 10 µL, 1 µL, and 0.1 µL ALGATAN™ dilutions was in the range 50.81 ± 1.15 - 81.25 ± 1.53; 37.21 ± 2.11 - 81.20 ± 1.38; 5.23 ± 1.69 - 67.56 ± 1.13; and 3.60 ± 0.89 - 51.88 ± 1.00.

There is a significant difference of antibacterial activity among the isolates and the ALGATAN™ dilutions tested (Table 3).

The highest percent bacterial were detected at 100 µL ALGATAN™ and they concerned mainly enteropathogenic *E. coli* isolates, *P. aeruginosa* and *C. perfrigens. C. perfrigen*s is drastically less susceptible to the other ALGATAN™ dilutions.

	Growth reduction (%)			
	Treatment			
Bacteria	**100 µL**	**10 µL**	**1 µL**	**0.1 µL**
Clostridium perfrigens ATCC® 13124™	76.19 ± 1.00 a	37.21 ± 2.11 a	5.23 ± 1.69 a	3.60 ± 0.89 a
Escherichia coli NLK99-3	81.25 ± 1.53 b	78.21 ± 1.20 b	67.56 ± 1.13 b	39.41 ± 1.08 b
E. coli CVVI KH10B	86.31 ± 1.15 c	81.20 ± 1.38 c	60.18 ± 1.30 c	38.86 ± 1.10 b
Staphylococcus aureus	50.81 ± 1.15 d	45.70 ± 1.00 d	42.48 ± 1.00 d	40.16 ± 1.14 bc
Streptococcus uberis	67.86 ± 1.00 e	66.19 ± 1.51 e	59.21 ± 0.50 c	51.88 ± 1.00 d
Pseudomonas aeruginosa	77.21 ± 1.00 a	65.73 ± 1.99 e	38.57 ± 1.11 d	22.36 ± 1.58 e
E. coli ATCC® 51813™	68.28 ± 0.87 e	47.51 ± 2.11 f	15.13 ± 1.69 e	19.90 ± 0.89 f
S. aureus ATCC® 29213™	62.34 ± 0.75 f	50.81 ± 1.20 g	47.36 ± 1.13 f	29.61 ± 1.08 g
S. uberis ATCC® 700407™	51.22 ± 1.00 d	51.20 ± 1.08 g	31.18 ± 1.30 g	26.56 ± 1.10 a
P. aeruginosa ATCC® 27853™	72.25 ± 1.18 g	66.13 ± 1.29 e	38.17 ± 1.00 d	22.46 ± 1.18 e

Table 3: *Bacterial growth reduction at 24 h in liquid medium with differences in the percentage of bacterial growth reduction at different dilutions of ALGATAN™ using the control treatment as reference (without ALGATAN™).*

Values expressed as the mean ± standard deviation of two experiments (three repetitions for each experiment). Mean values with different letters in the column are significantly different ($p \leq 0.05$).

Adhesive activity

Table 4 reports V_{50} values, the time at which half of the maximal growth yield reached as a measure for adhesion of the different bacteria. ALAGATAN™ dilutions with the lowest V_{50} (= the fastest appearance of bacterial growth) bound most cells of bacteria.

The highest binding activity were recorded at ALGATAN™ 100 µL and 10 µL dilutions towards the enteropathogenic *E. coli* NLK99-3, *C. perfrigens* ATCC® 13124™, *E. coli* CVVI KH10B, *S. aureus, and P. aeruginosa.*

	Bacteria* (V_{50})									
Treatment	**1***	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
BSA**	7.8^{a}	7.3^{a}	7.6^{cd}	7.7^{a}	7.5^{a}	6.8^{a}	7.7^{a}	7.6^{a}	7.8^{a}	7.3^{a}
ALGATAN™ 100 µL	5.8^{b}	6.6^{b}	4.8^{a}	6.3^{b}	6.6^{b}	5.6^{b}	7.0^{b}	7.0^{b}	6.8^{b}	6.3^{b}
ALGATAN™ 10 µL	6.7^{c}	5.6^{bc}	5.2^{abc}	6.7^{c}	7.1^{c}	6.7^{a}	7.0^{ab}	6.8^{c}	6.7^{b}	6.8^{c}
ALGATAN™1 µL	8.0^{ab}	7.5^{a}	6.1^{ab}	7.0^{d}	7.5^{a}	8.0^{c}	8.0^{c}	7.5^{a}	7.2^{c}	7.3^{d}
ALGATAN™ 0.1 µL	7.8^{a}	7.5^{a}	7.2^{abc}	7.3^{e}	7.8^{d}	7.3^{d}	7.8^{a}	7.8^{d}	7.1^{c}	7.5^{e}
Lsd***	0,4	0.3	0.4	0.5	0.4	0.3	0.3	0.5	0.2	0.3

Table 4: *Time [h] at which half of the maximal growth yield was reached (V_{50}) as a measure for adhesion of bacteria. 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 V_{50} (= the fastest appearance of bacterial growth) bound most bacterial cells. *1. C. perfrigens ATCC® 13124™; 2. E. coli NLK99-3; 3. E. coli CVVI KH10B; 4. S. aureus; 5. Str. Uberis; 6. P. fluorescens; 7. E. coli ATCC® 51813™; 8. S. aureus ATCC® 29213™; 9. S. uberis ATCC® 700407™; 10. P. aeruginosa ATCC® 27853™; ** Control; *** Least Significant Difference.*

The results in Table 5 represent the number of bacteria adhered at the start, as a measure for adhesion of the different bacteria.

The ALGATAN™ dilutions with the highest amount of bacteria bound most bacterial cells.

	Bacteria (Log_{10} count ml^{-1})									
Treatment	**1***	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
BSA**	2.8^{ab}	3.9^{a}	4.0^{cd}	2.6^{a}	2.6^{b}	2.8^{ab}	3.9^{a}	2.9^{a}	2.3^{a}	2.6^{a}
ALGATAN™ (100 µL)	3.8^{c}	4.1^{c}	4.5^{c}	2.9^{c}	2.0^{c}	3.4^{c}	3.6^{b}	3.6^{b}	2.6^{b}	2.9^{c}
ALGATAN™ (10 µL)	3.2^{a}	3.9^{a}	3.9^{a}	2.5^{a}	2.9^{a}	2.9^{a}	2.8^{ab}	2.8^{ab}	2.1^{ab}	2.5^{a}
ALGATAN™ (1 µL)	2.6^{b}	2.6^{b}	2.6^{b}	2.6^{b}	2.6^{b}	2.6^{b}	2.1^{ab}	1.7^{ab}	1.7^{ab}	2.6^{b}
ALGATAN™ (0.1 µL)	2.0^{ab}	1.7^{ab}	1.8^{ab}	2.1^{ab}	2.1^{ab}	1.8^{ab}	1.3^{bcd}	1.3^{bcd}	1.0^{bcd}	2.1^{ab}
Lsd***	0.3	0.3	0.4	0.5	0.4	0.3	0.3	0.2	0.5	0.4

Table 5: *Number of adhering bacteria to the plate coatings. The data represent least squared means. Data followed by different letters within one column are significantly different (P ≤ 0.05). Products with the highest Log_{10} count ml^{-1} bound most cells of bacteria. *1. C. perfrigens; 2. E. coli NLK99-3; 3. E. coli CVVI KH10B; 4. S.aureus; 5. Str. Uberis; 6. P.fluorescens; 7. E. coli ATCC® 51813™; 8. S. aureus ATCC®29213™; 9. S. uberis ATCC® 700407™; 10. P. aeruginosa ATCC® 27853™; ** Control; *** Least Significant Difference*

Biofilm dispersive activity

Data related to percent of Log_{10} biofilm reduction reported in Figure 1 show that ALGATAN™ act as biofilm dispersive agent of the matrix that results in the detachment of cells from the colony of all bacteria tested and their release into the plate's well. The highest biofilm dispersive activity were recorded using 100 µL and 10 µL of ALGATAN™ towards *E. col*i CVVI KH10B and *S. uberis* (4.5 %), *P. aeruginosa* (4.31 %) and *E. coli* NK99-3(4.3 %); *E. coli* CVVI KH10B (4.4%), *C. perfrigens* (3.31%), *P. aeruginosa* (3.1%), respectively.

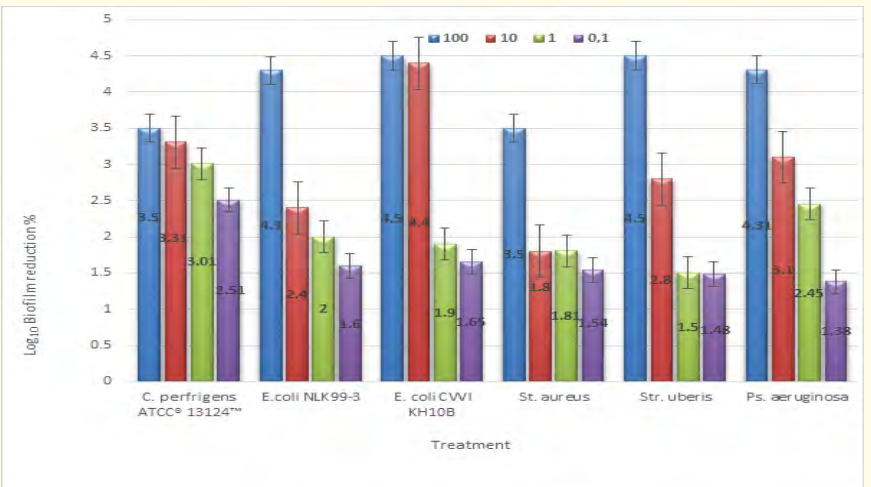

Figure 1: *Biofilm dispersive activity of ALGATAN™ at different dilutions (100 µL, 10 µL, 1 µL, 0.1 µL) towards C. perfrigens, E. coli isolates, S. aureus, Str. uberis and P. aeruginosa after 18h of incubation at 37°C.*

Anti-inflammatory activity

The ability of ALGATAN™ to inhibit COX-2 to catalyze the conversion of arachidonic acid to prostaglandin H2 (PGH2) was determined.

The results of the COX inhibition assay of ALGATAN™ at dilutions 100 µL, 10 µL, 1 µL and 0.1 µL listed in Table 6. The highest percent inhibitions were observed at 10 µL (COX1 33.2 ± 1.4; COX2 33.2 ± 1.4) and 1µL (COX1 39.2 ± 1.4; COX2 32.1 ± 0.9) product treatment.

Inhibition ± SD (%)[1] at dilutions of ALGATAN™						
Enzymes	**W[2]**	**100 µl**	**10 µl**	**1µl**	**0.1 µl**	**Rc[3]**
COX1	ns	17.4 ± 1.3	33.2 ± 1.4	30.6 ± 2.7	ns	38.4 ± 3.8
COX2	ns	36.3 ± 3.6	39.2 ± 1.4	32.1 ± 0.9	10.8 ± 0.5	48.7± 3.0

Table 6: *Effect of different ALGATAN™ dilutions on COX1 and COX2. 1 Values are the mean (n = 3) and SD of the % inhibition acquired using COX inhibition assay; 2 water. 3 Aspirin 1 mg/1mL water, V/V. ns = not significant (% inhibition < 10%).*

Conclusion

All the bacteria considered in the present work are environmentally ubiquitous. They are found in water, soil, common food, and tissues of plants and animals. They are also the major bacteria of nosocomial infection, very hard to eradicate, in part because of the intrinsic resistance of the bacteria against many different types of chemotherapeutic agents and antibiotics.

Today the antimicrobial resistance is a global emergency caused by improper use of the drugs, their indiscriminate use in farms and small investment of industries in the discovery of new antimicrobials, mainly due to high costs for their development. Currently, only 2.6% of potential antibiotics under study reached the human clinical trial, while their sales, which prices are also low, greatly diminished. It urges finding new antimicrobials able to constrain the growing risks, to strengthen the prevention and control of ever-increasing mechanisms of antimicrobial resistance in human, veterinary, food and to develop next-generation antimicrobials. Recently it was found a renewed interest in natural substances of plant origin as a potential reservoir of innovative therapeutic solutions to human, animal and plant health with the prospect of supplement and sometimes replace conventional medications. However, their potential is of big concern in the cosmetics industry, the nutraceutical, in food preservation and nutrition of pets and in animal production [33-35].

The use of anti- adhesion agents that interfere with the ability of the bacteria to adhere to surfaces including the host's tissues represents an attractive approach to constrain skin and mucosal infectious diseases in early stages to face increasing alarming antibiotic resistance worldwide [36-40]. Algal polysaccharides are potential innovative tools to treat bacterial infections. In fact, some possess antibacterial properties while others inhibit bacterial colonization by blocking specific carbohydrate receptors involved in host-bacteria interaction. This mode of action supported as alternative anti-adhesion therapy. Anti-adhesion agents do not affect the viability of microorganisms and thus do not destroy the human and animal associated healthy microbiota. Anti-adhesion agents expected to induce resistance at lower frequencies than antibiotics due to their mode of action.

Many natural polysaccharide compounds with anti-adhesive activity reported in scientific literature. However, until now only polysaccharides with anti-adhesion activity from *Aloe vera* developed into commercial anti-adhesive products, 2QR RESEARCH BV, NETHERLANDS and BIOCLIN B.V., NETHERLANDS, against *Helicobacter pylori, P. aeruginosa, Str. mutans* and *Str. sanguis* [41].

The introduction of anti-adhesion based product in livestock nutrition is of particular interest against diarrhea diseases [42].

New anti-inflammatory molecules that are efficacy, ecofriendly ad low cost needed to constrain degenerative syndromes and diseases with complex etiology that affect herds needed. These studies provide evidence to show that the ALGATAN™ exert an anti-inflammatory effect by inhibiting COX activity, which studied using an *in-vitro* model.

The use of herbs as new feed or remedies in herds should be in accordance with what declared in the label. This work show a laboratory approach for the assessment of the biological activities of a sheep and goat feed additive that claim antimicrobial and antinflammatory activities. All the obtained results, part of a preliminary screening program of plant raw material biological activities for zootechnics use, justify further *in vivo* tests.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Volume 6 Issue 3 February 2017