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Research Article

Prophylactic Effect in the Gut Microbiota After Oral Administration of HAMLET: Results of a Case Control Study

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Abstract

Cancer is one of the most common causes of death, with up to 14 million worldwide. The oncogenic therapies and immunotherapies against the diverse forms of cancer remain to be defined. Radio and Chemotherapy are still the anti-cancer of the first choice. However, the secondary effects of these treatments are detrimental to the health. Several studies have reported the pro-apoptotic properties of HAMLET (Human alpha-lactalbumin Made Lethal to Tumor cells). The hypothesis is that complex formation with fatty acids cause membrane disruption of the cancerous cells. Therefore, in the present study, we aimed to evaluate the oral administration of Hamlet in case control study. The microbial growth in stool samples of the individual treated with HAMLET at four different time points during two weeks were recording in selective medium (MC, Trypt, EMB, and BA) for Firm cutes and Bacteroidetes (90%) and for the rest 10% of mostly Gram negative), among them the Proteobacteria (of utmost of the family *Enterobacteriaceae*). Antibiotics sensibility and resistance, Gram staining and Colony-forming (CFUs) evaluated at different time points of the HAMLET treatment. Altogether, the data show a modulation of the composition and behavior of the gut microbiota in the treated than untreated individual. In EMB selectively grew in higher amount both types of colonies. From the results we suggest that the positive effect of the oral administration of HAMLET might be beneficial for patients before surgery and or radio/chemotherapeutic treatment.

Keywords: Hamlet; Cancer; Radiation Therapy

Introduction

Cancer is one of the most common causes of death. Up to fourteen millions worldwide [1]. cancer is a chronic disease characterized by unregulated cell division, leading to replicative immortality and resistance to cell death. Cancer cells grow into an abnormal cell mass, except in hematologic cancers, where cancer cells grow and spread through the blood, lymphatic systems and bone marrow. Cancer processes originate mainly from damage or mutation of proto-oncogenes that encode proteins involved in the induction of cell proliferation, differentiation, and tumor suppressor genes producing inhibitory signals of cell growth, stimulate apoptosis [2,3], angiogenesis involved in tumor growth and progression [4-7].

To control and eliminate cancer cells has been the subject of intense research for decades. Cancer patients undergoes chemotherapy treatments, radiotherapy, toxic compounds that mainly inhibit the rapid proliferation of cancer cells. In other cases, depending on the cancerous tumor, surgery may even be performed first [8-10] Chemotherapy based on toxic compounds inhibit the rapid proliferation of cancer cells. However, they can also inhibit the rapid growth of the cells necessary for the hair follicles, bone marrow, and gastrointestinal tract. This results in the undesirable side effects in cancer treatment [11,12]. Surgery and radiation therapy are the most effective and valuable treatments for local and non-metastatic cancers, but they are ineffective when the cancer has spread throughout the body. Current therapies against cancer of different types are based mainly in chemotherapies and radiotherapies which mainly inhibit the rapid proliferation of cancer cells [13-17]. In recent years, other alternatives based on natural products as Cannabinoids [11,12,18] are being reported. These natural chemical compounds can modulate proliferation, death of different cancer cells and angiogenesis [1,19], The Secondary effects of chemotherapy includes mucositis, anemia, long-term neutropenia, mutagenic changes, neuropathy or chronic heart failure [2,14,19].

Alpha-lactalbumin (α), a small (Mr 14,200), acidic (pI 4–5) Ca2+-binding protein, which mainly serves as the substrate of the lactate synthase, a component of lactose synthase enzyme system. α--LA is very important in infant nutrition since it constitutes a large part of the whey and total protein in human milk. Among other biological activities of alpha LA is in addition to its binding to fatty acids, such as oleic acid (C18:1) favoring a molten unfolded state, which endowed with antimicrobial and antiviral properties [20,21]. Moreover, the complexes of partially unfolded α--LA with oleic acid showed significant cytotoxicity to various tumor and bacterial cells [22-24]. As aforementioned above, alpha-LA plays a role as a delivery carrier of the cytotoxic fatty acid molecules (Oleic acid) onto the cell membrane of the tumor cells [22,25]. The oleic acid and the protein form a common core-shell structure, called lipoproteins (lipids and partially denatured proteins) considered as molten globular containers filled with the toxic oil which action initiates by stabilizing the unfolded protein in the complex protein: fatty acid followed by insertion and integration in the membrane [24,26] resulting in membrane disruption, internalization of (Human alpha-lactalbumin Made Lethal to Tumor cells) (HAMLET) and targeting cellular components and finally activation of different signalization pathways such as Apoptosis, Caspase, Ras, c-Myc pathways, and cell death [22,26-28]. In addition, of relevance is that in animal models, the therapeutic effect of HAMLET effect is defined by the expression of the oncogenes**.** The potential effect of HAMLET as anticancerigen is well documented in the literature [29-35] either in animal models with different types of cancer as well as *in vitro* studies, using cancer cell lines of different tissues [29-35]. Recent studies with peptides from alpha-LA [36,37] represent a hope and encourage for bladder cancer cells which usually therapeutic treatments has been failure or high rate of recurrence.. Taking advantage and the knowledge of the fact that partially unfolded alpha-LA, what is called the molten globular state, forms the oleic acid complex or HAMLET, with potent tumoricidal activity [36,37]. On referring to a clinical studies recently reported, a designed peptide of 39 amino acid residues of alpha helical conformation from alpha lactalbumin, complexed with oleic acid (alpha-1-oleate). This complex in the placebo controlled, double blinded Phase I/II interventional clinical trial of non-muscle invasive bladder cancer reached safety and efficacy. Furthermore, the treated tumors show evidence of apoptosis and the expression of cancer-related genes is inhibited [37].

By another hand, the diversity and the abundance of the gut microbiota imply a role in the human health [38] in the homeostasis of nutrients metabolism, and gut immunity [39;40]. The gut

is inhabited by thousands of microorganism estimated in 10^{14}, a mixed population of bacteria, archaea, fungi, and protozoa referred as gut microbiota. The role and contribution of the gut microbiota to the human health [40,41], because the diversity and abundance. The gut microbiome is composed utmost of ninety percent (90%) of Bacteroidetes and Firm cutes [42,43] (Figure 1 A). Ten percent (10%) includes to the Proteobacteria, Actinobacteria (Bifidobacterium), Fusobacteria, and Verrucomicrobia. Firm cutes are mainly Gram-positive bacteria, and mostly Clostridium (95%) and 5% are conformed by Lactobacillus, Bacillus, Eritrococcus and Ruminococcus. While the Bacteroidetes usually are Gram negative bacteria, such as Bacteroidetes and Prevotella [38]. Any disbalance onto the gut microbiota composition leads to disease in dysbiosis, which has been associated to inflammatory diseases such as, inflammatory diseases (IBD), inflammatory syndrome (IBS), as well as other chronic and autoimmune diseases (i.e. obesity, diabetes, asthma psoriasis, cancer, and neurological disorders [43,44]. The role and the contribution of the gut microbiota in the health axis, brain, gut microbiota is pointed to the fact that gut microbiota is endowed with the ability to synthesize a number of different nutrients elements that participate in the human metabolism. Among the vitamins (K,B, biotin, Cobalalimn, folate, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, and thiamin) [49,50]. Secondly, Furthermore, gut microbiota produces neurotransmitters causing good mood and cognition [44,45]. Besides, gut microbiota participate in promoting nutrient adsorption and metabolism through the degradation and fermentation of fibers, and the biotransformation of bile acids produced by the liver) [45-47]. Taking in account this knowledge and the secondary effects of the radio therapy/Chemotherapy side effects, in the present work we aimed to evaluate the effect of the oral intake of HAMLET, specifically on gut microbiota. To this end, microbiological analysis of the fecal microbiota was performed. The results obtained suggest that there is a probiotic like effect at the intestinal flora that could have a role to recover patient's mood and appetite.

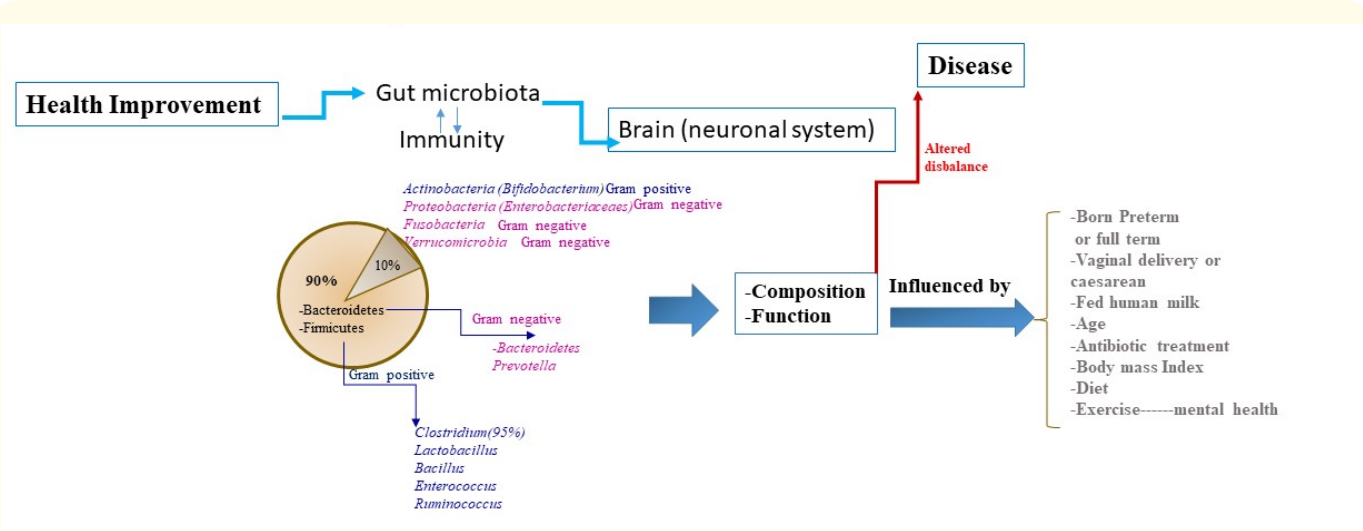


Figure 1A: The gut microbiota composition under normal conditions. Microbial diversity plays of utmost a role in the health and disease. Any disbalance in the composition can alter either the function of the microbiota. The composition can be influenced by intrinsic or extrinsic environmental factors, and therefore alter the function of the microbiota resulting in dysbiosis or another intestinal or neuronal disorder. B- Scheme of the protocol designed to analyze the effect of the oral intake of HAMLET in a period of two weeks. As depicted, Microbial growth, Gram staining, antibiotic resistance and unit forming colonies (CFUs) were recorded in each time point after oral intake administration of HAMLET (V= 30 ml).

Material and Methods

Study design and approval

The study and all the procedures for medical research involving human subjects, including research on identifiable human material and data were approved by the Ethic Committee in Research of the Zacatecas, General Hospital "Luz Gonzalez Cosio" CONBIOETICA-32-CEI-001-20180807. Healthy untreated individual and an Individual with affection and treated with HAMLET written informed consent. Adverse events and reactions were monitored systematically throughout the study.

Study procedures and timing of samples

The participant were instructed to take one dose of 30 milliliters of prepared HAMLET early in the morning every four days (Figure 1A) for two weeks. At each time point, feces and urine were taken for further clinical analysis. The participants were advised not take food before oral administration of HAMLET. Similarly, participants were instructed not to drink any probiotics during the study period.

HAMLET preparation

The complex of α-lactalbumine (LA):oleic acid (C18:1)(AO) was obtained from donors mother's milk. Thereafter was storage and frozen. During this time, human milk is acidified to reach a 15 Dornic (°D). The acidification process can be accomplished by adding ethylene diamine tetra acetic acid (EDTA).

Culture mediums

40 gr of Agar Middlebrook trypticasein (TCB Lab, Cat No. 7171) dissolved in 1000 ml of distilled water, with vigorous stirring, and boiling for 1 minute. Thereafter Bacteriologic Agar (MCD Lab Cat No 9011) was added at a concentration of 1.2 to 1.5%, w/w). The medium is sterilized at 121ªC (15 lbs.)/15 minutes and after medium reached a temperature of 451C, petri dishes were prepared. The medium, Agar Eosins and methylene blue (EMB)(MCD Lab, Cat No 7051)(35 g/ lt); (MacConkey (MCD LAB, Cat No 7111)(50/1t) and the medium Base Blood Agar (MCD LAB, Cat No 7241)(40 gr/1 Lt).

Microbial growth, and composition

A sample of feces were resuspended in 1 ml of sterile water, and vortexed for homogenization. An aliquot of 100 microliters were spread into solid medium of Agar eosin and methylene blue (EMB) (MCD Lab, cat no 7051), MacConkey (MCD LAB, cat no. 7111); trypticasein broth (TCB, MCD Lab, cat no 7171); Bacteriologic agar (no de cat 9011), and Base Blood Agar (MCD LAB, cat no. 7241). The plates were incubated at 37ªC for 48 h. Growth was recorded in each medium, by plate counting of the serial dilutions. The counting was expressed as the log of colony forming units (CFUs). A general identification of the microbial growth was assessed by Gram Staining KIT (HYCEL GRAM DYES, cat no.541), following the manufacturer's instructions. Antibiotic susceptibility/resistance assessed with sensidiscs (DT-35 Multibarc LD, cat no; 6P1 DT-34, cat no. 6P1).

Statistical analyses

Statistical analyses were performed using Graph Pad Prism 6.0 (CA, US) using a non-parametric analysis of variance (ANOVA). A $p \leq 0.05$ was considered significant

Results

Prophylactic effect of the oral intake of HAMLET.in a case control study

The effect of the oral intake of HAMLET (Dorninc of 11 to 12) was analyzed in a case control study. To this end a protocol was designed (Figure 1B). As depicted in the scheme, a period of two weeks with intervals of four days were accomplished. In each time point starting from zero (written consent informed signed). Next day, the stools were recollected before the first dose (time= 1) of HAMLET (V= 30 ml). After four days' time = 2), the second dose of HAMLET, stools were collected. The third dose of HAMLET after four days (time= 3). The fourth dose after four days (time = 4) - Stools collected before the fourth dose [38,41-47].

The microbiological analysis of the fecal microbiota were divided in four aspects: microbial growth in different and selective media cultures (Mat and Methods). Secondly, Gram staining for identification of Gram Negative and Gram Positive. The third point was to evaluate the sensibility and resistance to antibiotics. The fourth point was to count the colony units forming (CFUs) in the different media.

Fecal Microbiota growth

For the characterization of the microbial growth in different selective media, stools were homogenized as described in Materials

and Methods. From the Figure 2, healthy untreated individual (A) and treated individual (B), it is observed that bacteria grew at the different time points and in the different media cultures, with a diverse morphology. In MacConkey (MC) media the colonies were rose dark colored, while in Eosin Methylene Blue Agar (EMB) the colonies for one side green brilliant and other were dark blue (A). In (B), the colonies were also of similar color in MC but the color of the medium were changed in all time points (1-4) while in EMB the colonies were mostly dark blue and in others looks like drops of Bougainville color (time point 3). In addition the fecal microbiota growth in B changed also the color of the medium. While the colonies of the fecal microbiota seeded in trypticasein soy agar and Blood gar (BA) utmost are cream colored colonies at each time point in A and in B.

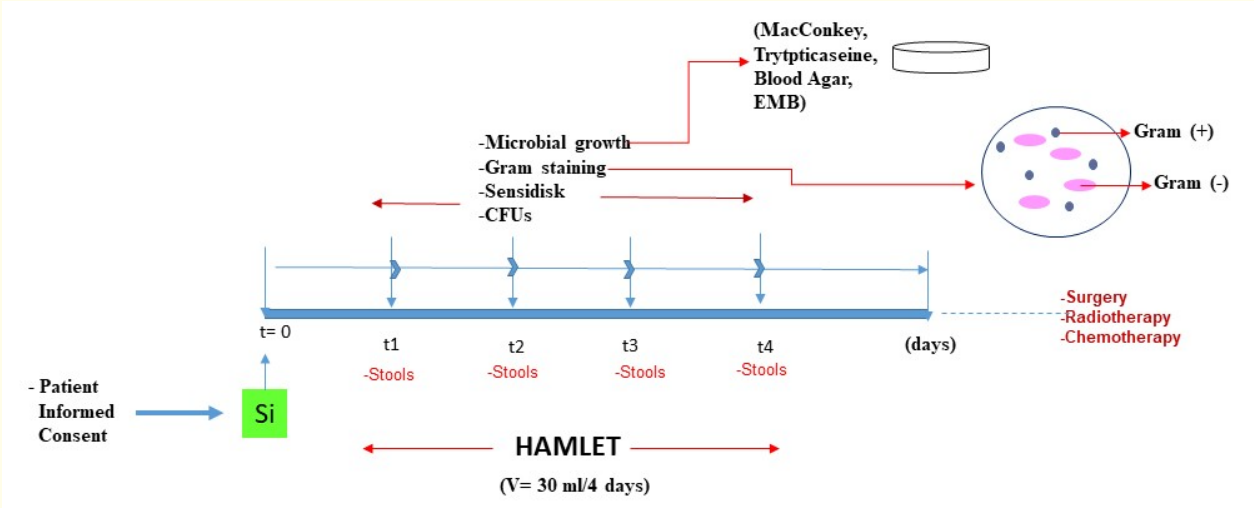


Figure 1B: Microbial growth of fecal microbiota in selective medium. Stools from the healthy individual (A) and the individual under the treatment of HAMLET at each time point (1-4) were homogenized in sterile water and seeded in selective medium [MacConkey, Trypticaseine soya agar, Blood Agar (AS) and Eosin Methylene Blue Agar (EMB)] as described in material and Methods. In MacConkey (Mc) and Eosin Blue methylene agar (EMB) medium different colorful colonies than the colonies that grows in Trypticaseine soya agar (Trypt) and Blood Agar (AS) where usually cream colored colonies grew. A change of the medium culture (EMB and Mc) induced by the individual treated with HAMLET (B) was more evident than in healthy individual (A).

Fecal Microbiota growth are mostly Gram negative Bacteria

The gut microbiota is predominantly *Firm cutes* and *Bacteriodetes* (90%) mostly Gram positive bacteria while the rest of the bacteria are Gram negative, between them the phylum of *Proteobacteria*, the gamma group comprised by the *Enterobacteriaceae* [38-47] (Figure 1A). From the stool samples (Mat and methods), at each time point (1-4). From Figure 3, on the right hand (A) untreated individual and the left hand (B) treated individual. Thus, in A, it is observed that fecal microbiota seeded in MC analyzed at 100X, times 1 and 3, a mixed population of Gram negative and Gram positive while in times 2 and time 4, mostly are Gram negative. In Trypt, a mixed population of Gram negative and Gram positive at times 1, 3, and 4 while at time 2 mostly are Gram negative. Moreover, fecal microbiota seeded in BA, and EMB, mostly of were Gram-negative at all time points, except that at time point 4, it is observed a mixed population of Gram negative and Gram positive. In treated individual con HAMLET (B), the fecal microbiota seeded in MC, at time 1 and 3 a mixed population of Gram positive and Gram negative while at times 2 and 4 predominantly Gram negative were observed. In Trypt, the fecal microbiota that grew were mostly Gram negative bacteria in all the time points, (1-4). In BS, at time 1 and 2 a mixed population of Gram negative and positive. At times 3 and 4 predominantly grew Gram positive. In EMB, at times

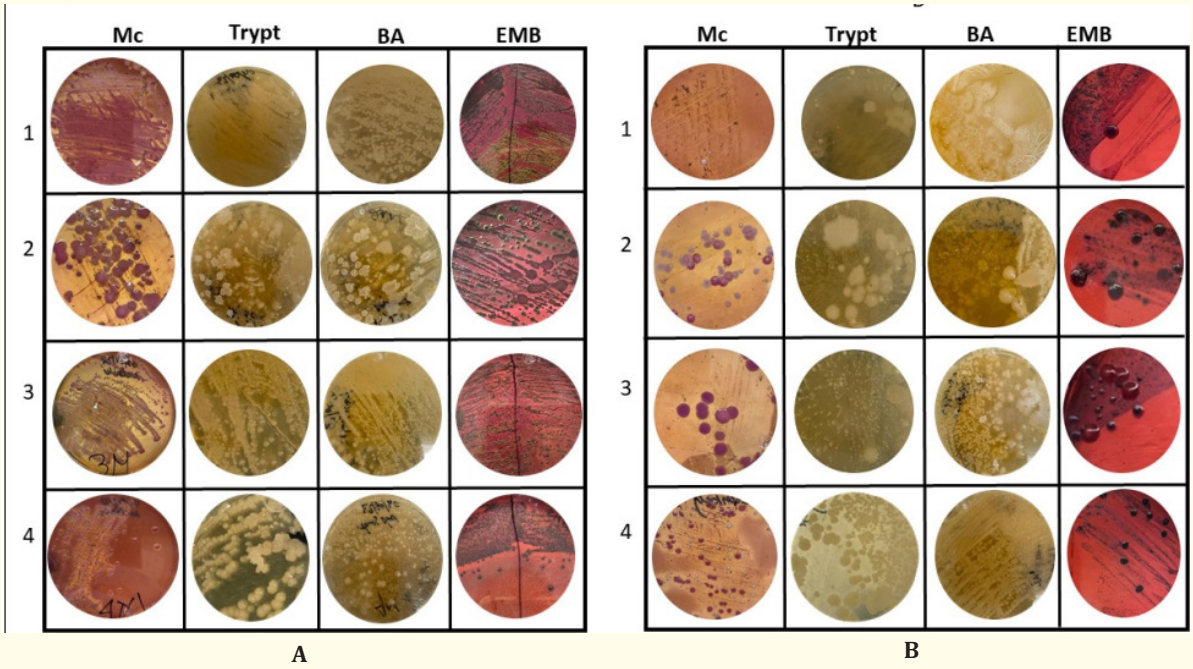


Figure 2: Gram staining of the fecal microbiota. The analysis of the effect of the oral intake of HAMLET in the gut microbiota at each time point (1-4) microbial growth in each of the medium were processed for Gram staining. It was found that mostly are Gram negative, coincident with the Entero bacteria presence. The fecal microbiota of the individual under the HAMLET treatment after the first intake of HAMLET a mix of Gram negative and Gram positive was observed, however after the fourth dose, utmost Gram negative were observed. While in the healthy individual a mix of Gram negative and Gram-positive bacteria (mixed staining between dark blue and pink). A representative image at 100X of at least five fields in the optical microscopy.

1,3 and 4 mostly Gram negative while at time 2 a mixed population of Gram negative and Gram positive bacteria.

Susceptibility and Resistance of the fecal microbiota after oral ingestion of HAMLET

To evaluate the effect of the oral ingestion in the susceptibility or resistance to antibiotics of the Gram negative and Gram positive identified above, fecal microbiota was seeded in each of the medium cultures at each time points. From Table 1A (Gram-positive) and Table 1B(Gram-negative), the growth (resistance) and the inhibition of this (susceptibility) in the presence of the different antibiotics outlined at the right side indicated respectively as low (+) or medium (++) or high (+++) respectively from untreated (H) or treated individual (P).

Of relevance is that the expression of the sensibility and the resistance is influenced by the selective medium culture. The Gram positive bacteria of untreated individual (Table 1A) showed in MC showed at times 1 and 3 a susceptibility to the different antibiotics, At time 2 a mixed S/R trait. At time 4 mostly a resistance to the different antibiotics. In treated individual, at time 1 a susceptibility to the antibiotics, while at time 2 resistance, at time 3 a mixed S/R and at time 4 mostly a resistance to the different antibiotics. In Trypt, untreated individual showed a resistance to the antibiotics at all time points while Gram positive bacteria of treated individual showed at time 1and 4 resistance, and at time 2 and 3 a mixed a mixed S/R to the different antibiotics. In medium BS, Gram positive bacteria of untreated individual at times 1, 3 and 4 resistance to the different antibiotics. At time 2 a mixed S/R to the antibiotics. Gram positive bacteria from treated individual showed at times 1 a mixed S/R to the different antibiotics. At time 2 resistance to the antibiotics and at times 3 and 4 mostly susceptibility to the different antibiotics. Finally, Gram positive grew in EMB of untreated individual at time 1 and 3 showed a susceptibility to the antibiotics,

while at times 2 and 4 a resistance to the different antibiotics. The Gram positive bacteria of the treated individual at all times showed mostly a susceptibility to the different antibiotics (Table 1Aa).

-Gram negative from untreated individual (H) in MC at time 1, 3 and 4 showed mostly susceptibility while at time 2 a mixed trait of susceptibility and resistance. In Trypt, Gram negative bacteria showed mostly susceptibility trait at time points of 1-3 while at time 4 mostly a resistance trait (Tables 1Aa). In BS, untreated individual, at time 1 and 3 a mixed trait S/R, while at times 2 and 4 mostly a susceptibility trait. In EMB medium, the Gram negative bacteria of untreated individual showed susceptibility to the different antibiotics at all time points (1-4). In contrast, Gram negative bacteria from treated individual showed mostly resistance to the different antibiotics at times 1 and 2, while at time 3 a mixed trait S/R and at time 4, mostly the Gram negative bacteria showed susceptibility to the different antibiotics (Table 1B, 1Bb) Moreover, treated individual (P) the Gram negative bacteria in MC showed at time points 1 to 3 a mostly susceptibility trait, however at time point 4 showed mostly a resistance trait (Table 1B). In Trypt, Gram negative bacteria from treated individual (P) showed a similar trait than untreated individual (H) except that at time 4 mostly showed a susceptibility trait. In BS, at time 1 mostly Gram negative were susceptible than resistance, at times 2 and 3 mostly Gram negative showed resistance to the different antibiotics. At time 4, Gram negative showed mostly susceptibility to the different antibiotics. Gram negative bacteria in EMB from treated individual showed mostly resistance to the different antibiotics at times 1 and 2, while at time 3 a mixed trait S/R and at time 4, mostly the Gram negative bacteria showed susceptibility to the different antibiotics (Table 1B, 1Bb).

Increase in the colony forming units (CFUs) after oral intake of HAMLET

To evaluate the effect of the oral administration of HAMLET in intestinal flora (decrease or increase), counting of the colony forming units (CFUs) were recorded at the different medium, and at each time points in untreated (A) and treated individual (B) (Figure 4I-4IV). CFUs counting of the two different colonies were made. In untreated individual, CFUs in MC (Figure 4A, I) of the small colonies (Bougainville) were slightly lower than the big colonies at times point of 1 and 2, equal at time point 3, and lower at times point 4. In Trypticasein (Figure 4A, II), a similar pattern was observed, specifically the proportion of CFUs of small colonies (cream colored) was lower than big colonies at time 4. In BS (Figure 4A, III), were higher than the small colonies (cream colored) at times point 1, 2 and 4 except at time point 3 where there not difference. CFUs in EMB (Figure 4A, IV) grew only one type of colonies, counted at a dilution of 10-5. CFUs were higher at time points of 2 while at times point 1, 3, and 4 were lower. In Treated individual, CFUs in MC (Figure 4B, I) of the small colonies (Bougainville) and big colonies (blue) were very similar in number at all time points. In Trytpcasein (Figure 4B, II), the number of CFUs of the small colonies were higher at time points of 1, 3 than at times points of 2 and 4. However, in comparison with the untreated individual the CFUs were higher. The big colonies increased from time points 1 to 3 and a slightly decrease at time point 4 but still the CFUs were higher than untreated individual. In BS (Figure 4B, III), an increase of big colonies at time point of 1 and 2, and a slightly decrease at time points of 3 and 4, slightly higher than time point 1. The CFUs of the small colonies increase from time point 1 to time points 2-4. Interestingly, the number of CFUs of the small colonies were higher at times points 3 and 4 than untreated individuals (Figure 4A. III).

Furthermore, in EMB (Figure 4B, IV) two types of colonies were counted, Boungaiville, and dark blue. At time point of 1 there is a higher CFUs of both types of colonies than in time points of 2-4. In comparison with the untreated individual (Figure 4A, IV), the dark blue colonies were lower in amount than in treated individual at all times points (Figure 4B, IV).

Discussion

The aim of the present study is to evaluate the potential positive effect of the oral administration of HAMLET (Human alpha-lactalbumin Made Lethal to Tumor cells) in an individual with a chronic affection. The results obtained suggest that oral administration of HAMLET might have a positive effect in the composition and function of the gut microbiota causing improvement in the appetite and in the mood of the patient(s).

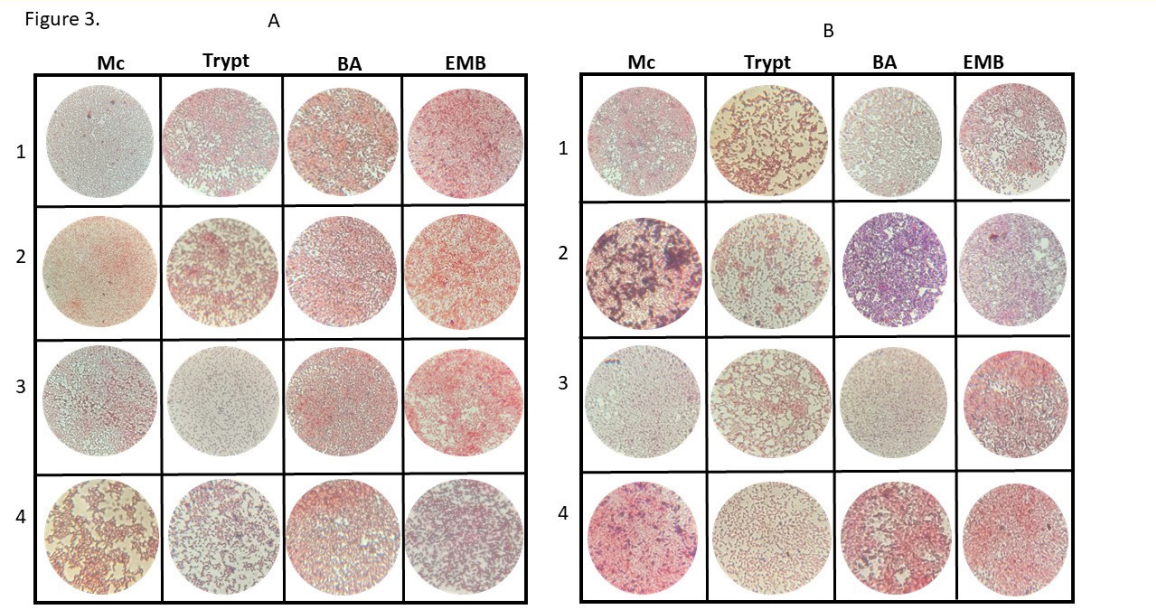


Figure 3: Colony Units Forming (CFUs). The analysis of the CFUs in each of the medium at each time point (1-4) at dilution of 10^{-5} rendered a pattern of grow similar between the healthy individual and the individual (A) under the treatment with HAMLET (B). However, the balance between the small colonies (orange) and the big colonies (blue) were more consistent in B in all the medium tested (I-IV) (Mc, Trypt, BA, EMB). In addition, in EMB (IV) the purple and the bougainvillea colonies were more predominant at 10-5 than in healthy individual. At $P < 0.05$ between time points of each medium as well as between big (blue) and small colonies (orange) were considered significant.

Alpha-lactalbumin (α) (alpha-LA)a small (Mr 14,200), acidic (pI 4–5) Ca2+-binding protein, which mainly serves as the substrate of the lactate synthase, a component of lactose synthase enzyme system [20]. Remarkable, several reports have highlighted that the complexes of partially unfolded α--LA with oleic acid showed significant cytotoxicity to various tumor and bacterial cells [22,25]. The oleic acid and the protein form a common core-shell structure, called lipoproteins (lipids and partially denatured proteins) considered as molten globular containers filled with the toxic oil which action initiates by stabilizing the unfolded protein in the complex protein: fatty acid followed by insertion and integration in the membrane, resulting in membrane disruption, internalization of HAMLET and targeting cellular components and finally activation of different signalization pathways such as Apoptosis, Caspase, Ras, c-Myc pathways, and cell death [22,26-35]. Furthermore, the bactericidal and antiviral properties of alpha-LA and its fragments are documented in the literature. Interestingly trypsin and chymotrypsin digestion yields peptides with bactericidal properties [48]. Fragments of alpha-LA obtained after endopeptidase digestion (pepsin and trypsin) are capable to lower the blood pressure in hypertensive adult rats [49]. In another study, a long peptide of 35 amino acid residues cleaved by endopeptidases in the residues 59-93 induces the growth of human fetal lung fibroblast cells [50]. Moreover, in addition to the above biological activities of the alpha LA, this molecule possesses remarkable immunomodulation activities, either in a native and hydrolyzed state. In the murine models it has been shown that alpha-LA enhance the antibody response to systematic antigen stimulation [51] and have a direct effect on the cellular immune response, specifically on B lymphocytes function and it is able to suppress T cell dependent and T cell independent cellular responses [52]. Of utmost importance are the studies in animal models where oral administration of alpha-LA has been evaluated. The observed effects goes from; (i) inhibition of writh-

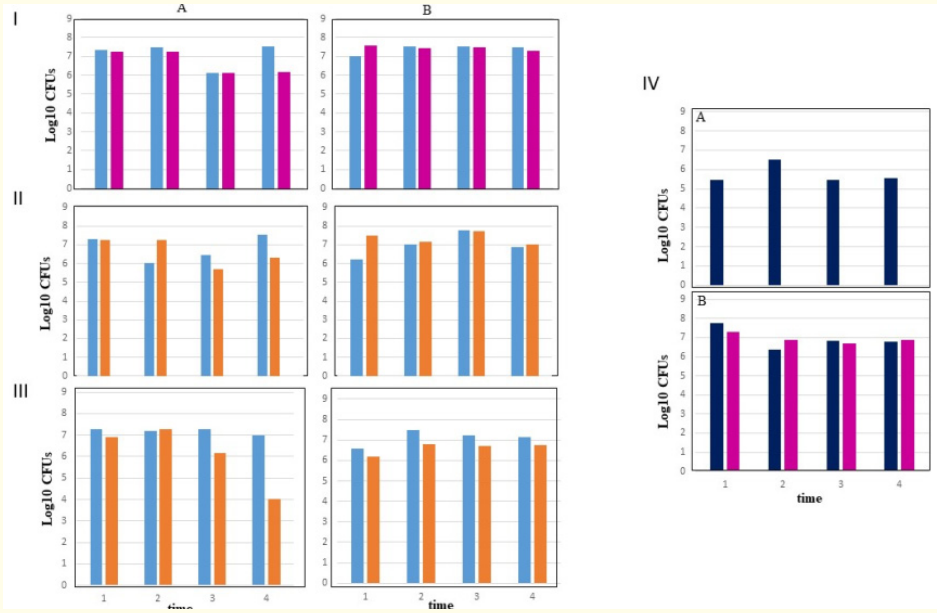
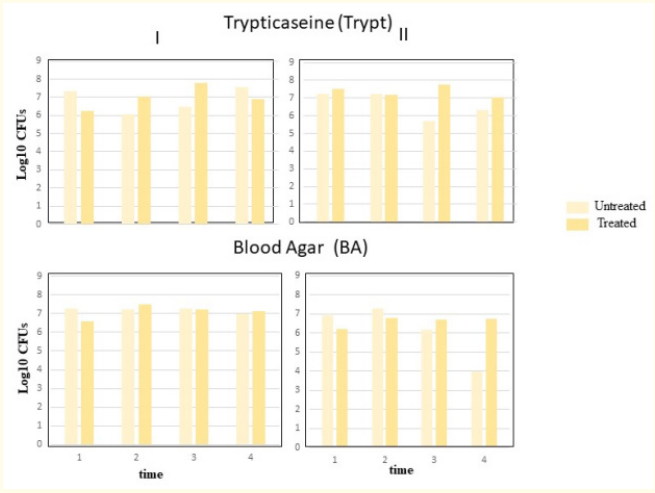


Figure 4: (CFUs) Very important



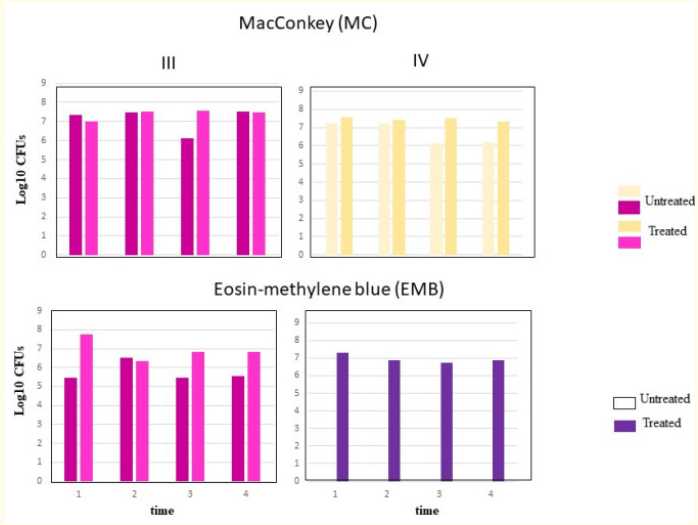


Figure 4: (CFUs) is the same as 5 but with graphics separated and with different color bars d

ing induced by acetic acid (mice); in (ii) suppression of nociception and inflammation in rat footpads, and (iii) a therapeutic effect on the development of adjuvant-induced pain and inflammation in rat [64]. iv) The administration of alpha-LA 1 h before carrageenan injection inhibited the increased formation of interleukin-6 (IL-6), and prostaglandin 2 in paw exudates. v) alpha-LA inhibited cyclooxygenase and phospholipase A2 activities *in vitro,* and vi) alpha -LA has a marked suppressive effect on hepatic fibroses through a nitric oxide-mediated mechanism in rats [53]. In addition, treatment of RAW 264.7 cells with a high concentration alpha-LA (100 g/mL) results in their time and dose-dependent decrease in either growth activity, morphological changes, increase in hypo diploid DNA population, and DNA fragmentation [54]. In fact, a high dose of this protein induces cellular apoptosis and necrosis. It activates several signalization pathways, such as the cytochrome c, active caspase 3, active caspase 8, extracellular signal-regulated kinase (ERK1/2) and c-Jun N-terminal kinase (JNK) activation however suppresses the protein level of Bcl-2. Indeed it has been suggested that long term consumption of alpha-LA reduce the risk of colon cancer [55], possible through a inhibition of cyclooxygenase-2 [55]. Of relevance it the finding that even a single exposure to the culture medium containing alpha-LA of an active lot for a period as short as 30 min is enough to provoke cell death, possibly through apoptosis [55,56]. In fact the potential mechanism could involve a direct effect at the nuclear level [22]. Interestingly, HAMLET in tumor cells co-localizes with histones H3, H4, and H2B [56-59], and perturbs the chromatin structure, impairing their deposition on DNA. The interaction of HAMLET with histones and chromatin in tumor cell nuclei locks the cells into the death pathway by irreversibly disrupting chromatin organization. Alpha-LA internalizes into the cells and enters even the nucleus only when it is complexed with oleic acid [55,56].

By another hand, on referring to the role of the gut microbiota in health and disease. Specifically referring to mental health (brain) and in chronic diseases such as cancer. In mice, it has been studied that even a short exposure to psychological stressors, is possible to modify the gut microbiota resulting in a reduction of *Lactobacillus* [38-47]. The presence of this bacteria has been associated with health benefits, immunomodulation and reduction of inflammation [60]. Moreover, some neuropeptides produced in the gut have displayed antimicrobial activity. In addition, hormones such as adrenaline, noradrenaline, and cortisol produced through activation of the hypothalamic–pituitary–adrenal axis have been correlated with bacterial pathogen's growth [61]. Furthermore, it has been found that colonization of ice with microbes could have a role in the regulation of emotions, could even increase anxiety [62]. This can be likely attributed to the e bi-directional interaction between the central nervous system and the enteric nervous system via the vagal nerve, commonly known as the gut-brain axis. However, it has been suggested that it could be also due to the neuropeptides generated by endocrine cells and the neurotransmitters potentially produced by gut bacteria [61,63]. Thus, Gut microbiota could also have an influence in the central nervous system thorough the production of short chain fatty acids (SCFAs) which has been shown influence epigenetic regulation linked, in turn, to the development of brain and behavior [62,63]. Besides, it is also believed that these SCFAs may affect the permeability of the gut epithelium, and reach the brain through the bloodstream, affecting it directly [61-64]. In another study on mice it was found that highly caloric diets rich in fat and sugar could be linked to changes in microbial composition that adversely affect mood [61-64]. From our microbiological analysis of the fecal microbiota, we have found that the oral administration of HAMLET have an effect in the modulation in the susceptibility and resistance to the different antibiotics (Table 1A, 1Aa), Moreover the Gram negative bacteria that grew in MC and in EMB there is a change from Resistance to Susceptibility trait to the different antibiotics (Table 1B, 1Bb). Not difference with respect to untreated individuals at any time points in BS and in Trypt (Table 1Bb). Of relevance is that the CFUs measured at all time points (1-4) and in the different mediums (Figure 4 I-IV) were more consistent at all time points and higher than in the untreated individual. Therefore, all the data together support the notion that gut microbiota and the oral administration of HAMLET in an individual with a chronic disease (i.e. cancer) could have a role in the improvement of the appetite and the mood (personal communication). Finally, it can be concluded that HAMLET remains as a potential anti cancerigen and utmost as a candidate for prophylactic cancer therapies, that deserve deep insight of clinic evaluation under different settings.

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Declarations Statement

The authors declare no conflict of interest.

Table 1A: Antibiotic susceptibility and resistance screening in Gram-positive bacteria isolated from stools of treated individual with HAMLET

		MacConkey								Trypticaseine								Blood Agar								Eosin-Methylene Blue							
		Time of treatment(d)								Time of treatment(d)								Time of treatment(d)								Time of treatment(d)							
		1		2		3		4		1		2		3		4		1		2		3		4		1		2		3		4	
Antibiotic		R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Ampicilin	**H**	+++			+++		+++	+++		+++		+++		+++		+++		++	+	++	+	+++		+++			+++	+++		+++			+++
	P	++	+	+++		+++		++	+	+++			+++	+++		+++		+++		+++		++	+	+++			+++		+++		+	++		+++	
Cefalottin	**H**		+++		+++		+++		+++		+++	+	++		+++		+	++			+++		+++		+++	++	+	+++		+	++		+++	
	P	+++		++	+	+++		+++		+++			+++	+++		++		+++		+++		+++		+	++		+++		+++			+++		+++	
Cefotaxime	**H**	+++			+++		+++		+++	++	+	++	+	++	+	+	++	+++		+++	+	+++			+++	++	+	+++		++	+		++	+
	P		+++	++	+	+++		++	+		+++	+++			+++		+++		+++	+++			+++	++	+		+++		+++		++	+		+++	
Ciprofloxacin	**H**	+++			+++		+++		+++	+++		+	++	++	+		+++	++	+		+++		+++		+++	+	++	+	++		+++		+++	
	P		+++	+++		+++		++	+		+++		+++		+++	++	+		+++	+++		++	+		+++		+++		+++		+++		+++	
Clindamycin	**H**	+	++	+	++	+++		+++		+++		+++		++	+	++	+	+++		+	++	+	++		+++	+	++		+++		++	+	++		
	P	+	++	+++		++	+	+++		+++		+++		+++		+++		++	+	+++		+++		+	++	+	++	+++		+++		++	+		
Dicloxacillin	**H**	+++		++	+	+	++	++	+	+++		++	+	+++		+	++	+	++	+	++		+++	+++		+	++	+++		+++		+	++		
	P	+++		+++		+++		+++		++	+	+++		++	+	++		+++		+++		+++		+	++		+++		+++		++	+		
Eritromycin	**H**	+++		++	+		+++		+++	+++		+++		+++		+++		+++		++	+	+++		++	+	++	+	+	++		+++	+++			
	P	++	+	+++		+++		+++		+++		+++		+++		+++		+++		+++		+	++	+++		++	+	+++		+	++		+++		
Gentamicin	**H**	+++			+++	+++		+++		+	++	+	++	+++			+++	+++		+++		+	++	+++		+++		++	+	+	++	+++			
	P	+++		++	+	+++		+++		+++		+++		+++		+	++	+++		+++		+++		+++	+	++		+++		+++			+++		
Penicillin	**H**	+++		++	+	+++			+++	+++		+++		+++		+++		+++		+++		+++			+++		+++		+++			++	+		
	P	+++		+++		+++		+++		+++		+++		+++		+	++	+++		+++		+	++	+	++	++	+	+++		+++		+	++		
Tetracycline	**H**	+	++	+++		+	++	+++		+++		+++		++	+	++	+	++	+	+++		+++		++	+	+++		+++		+++		++	+		
	P	+++		+++		+++		+++		+++		+++		+++		+++		+++		+++		+++		++	+	+++		+++		+++		++	+		
Sulfamethoxazole /Trimethoprim	**H**	++	+		+++		+++	+++		+	++	+++		+++		+++		+	++	+	++		+++		+++	++	+		+++		+++	+++			
	P		+++	+++		+++		+++		+++		++	+	+	++	+++		+++		+++		+++		++	+	+++		+++		+++		++	+		
Vancomycin	**H**		+++	+++		+	++	+++		+++		+++		+++		+++		++	+	+	++	+++		+++		+++		+++		+	++	+++			
	P	+++		+++		+	++	+++		+++		+	++	+++		+++		+	++	+++		+++		+++		+	++	+	++	+++		+	++		

Nota. H: Healthy individual. P: Problem. S: Susceptibility. R. Resistance, indicated as +/`+; ++/++; +++/+++ from low to higher growth or not growth.

Table 1Aa: Antibiogram of Gram-positive bacteria.

	MC		Trypt		BA		EMB	
Time of treatment	**H**	**P**	**H**	**P**	**H**	**P**	**H**	**P**
1	S	S	R	R	R	S/R	S	S
2	S/R	R	R	S/R	S/R	R	R	S
3	S	S/R	R	S/R	R	S	S	S
4	R	R	R	R	R	S	R	S

Nota. H: Healthy individual. P: Problem. S: Susceptibility. R; Resistance. MC. Maconkey; Trypt, trypticaseine; BA, Blood Agar; EMB, Eosin-Methylene Blue. Summary of data from table 1A.

Table 1B: Antibiotic susceptibility and resistance screening in Gram-negative bacteria isolated from stools of treated individual with HAMLET.

		MacConkey								Trypticaseine				Blood Agar				Eosin-Methylene Blue			
		Time of treatment								Time of treatment				Time of Treatment				Time of treament			
		1		2		3		4		1		2		3		4		1		2		3		4	
Antibiotic		R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Amikacin	H	+++			+++		+++		+++	+++		+	++		+++		+++	++	+	+	++		+++	+	++
	P	+++			+++		+++		+++	+++		+	++		+++		+++	+++		++	+	+	++		+++
Ampicilin	H	++	+	+++		++	+	++	+	+++		+	++	+++		+++		+++		++	+	+++			+++
	P	+++		++	+	++	+	+++		+++		++	+	+++		+++	+++	+++		++	+	+++		+++	
Carbenicilin	H	+++			+++	++	+	+++		+++		++	+	++	+	+++		+++		+	++		+++		+++
	P	+++		+++		+++		++	+	++	+	++	+	+++		+++		+++		++	+	+	++	+++	
Cefalotin	H	+++		+	++	+++		+++		+	++		+++		+++		+++	++	+		+++		+++		++
	P	+++		+++		+++		+	++	+	++		+++		+++		+++	++	+		+++		+++	+++	
Cefatoxim	H	+++		+	++	+	++		+++	+++			+++		+++		+++		+++	++	+		+++		+++
	P	+++		+++		++	+		+++	+++		+	++	+	++		+++		+++		++	+	+	++		+++
Cyprofloxacin	H	+++			++	+	+++		+++	+++		+	++		+++		+++	+	++		+++		+++		+++
	P	+++		++	+	++	+		+++	+++			+++	+	++		+++		+++		+++		+++	+	++
Chloramphenicol	H	+++		+	++		+++	+	++	+++			+++		+++		+++		+++		+++		+++		
	P	+++		+	++	+	++		+++	+++			+++		+++		+++		+++	++	+		+++	+++	
Gentamicin	H	+++		+	++		+++		+++	+++			+++		+++	++	+	++	+		+++		+++		+++
	P	+++			+++		+++	++	+	+++		++	+		+++		+++		+++		+	++		+++		+++
Netilmicin	H	+++		++	+		+++	++	+	+++			+++		+++	++	+	+++			+++		+++		+++
	P	+++			+++		+++		+++	+++			+++	+	++		+++		+++	+	++		+++		+++
Nitrofurantoin	H	+++			+++		+++		+++	+++			+++	+	++	+++			+++		+++			+++	
	P	+++		+	++		+++		+++	+++			+++		+++		+++	++	+		+++	+	++		+++
Norfloxacin	H	+++			+++		+++		+++	+++			+++		+++		+++	+	++		+++		+++		+++
	P	+++			+++		+++		+++	+++			+++		+++		+++		+++		+++		+++		+++
Sulfamethoxazole /Trimethoprim	H	+++			+++	+	++		+++	++	+	++	+		+++	+++		++	+	+	++		+++		+++
	P	+++			+++		+++		+++	+++			+++		+++		+++		+++		+++		+++		+++

Nota. H: Healthy individual. P: Problem. S: Susceptibility. R. Resistance, indicated as +/`+; ++/++; +++/+++ from low to higher growth or not growth

Table 1Ba: Antibiogram of Gram-negative bacteria.

	MC		Trypt		BA		EMB	
Time of treatment	**H**	**P**	**H**	**P**	**H**	**P**	**H**	**P**
1	S	S	S	S	S/R	S	S	R
2	S/R	S	S	S	S	R	S	R
3	S	S	S	S	S/R	R	S	S/R
4	S	R	R	S	S	S	S	S

Nota. H: Healthy individual. P: Problem. S: Susceptibility. R; Resistance. MC. Maconkey; Trypt, trypticaseine; BA, Blood Agar; EMB, Eosin-Methylene Blue. Summary of data from table 1A.

Ethics Approval and Consent to Participate

The study and all the procedures for medical research involving human subjects, including research on identifiable human material and data were performed under the principles of the Declaration of Helsinki, and approved by the Ethic Committee in Research of the Zacatecas, General Hospital "Luz Gonzalez Cosio" CONBIOETICA-32-CEI-001-20180807.

Consent for Publication

Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review.

Competing Interests

The authors declare no competing of interests.

Availability of Data and Material

Data will be shared following institutional guidelines. The review of the literature was based on search and data from PubMed database without limitation to 2024.

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Author's Contributions

G.G.G.M. and A.A.C. conceptualization, G..G.G.M. methodology, analysis. and writing. D.C.R.M. D.C.S. P.R.M. collaboration in patient's contact, clinic lab samples analysis, discussion. A.E.T. methodology. All authors have read and approved the manuscript.

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