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Influence of Iron Chelating Agents on the *in Vitro* Growth Curve of *Corynebacterium pseudotuberculosis* Strains

Influência de Agentes Quelantes de Ferro na Curva de Crescimento *in Vitro* de Cepas de *Corynebacterium pseudotuberculosis*

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Abstract

Corynebacterium pseudotuberculosis is the causative agent of caseous lymphadenitis in goats and sheep, a disease associated with direct economic losses in the livestock sector. In addition to having a chronic course, its treatment with antibiotics is unfeasible and there are no effective vaccines available on the market. In this experiment, *in vitro* culture conditions that approximated the environment to the *in vivo* conditions found by the bacterium during host infection were used, this included the use of animal biological material as a culture medium and the restriction of free iron in the medium. Under these conditions, *C. pseudotuberculosis* was expected to express unknown membrane protein profiles yet. Also, bacterial strains of low (T1) and high (VD57) virulence were used, grown in BHI broth, MQD, MQD supplemented with iron, RPMI, RPMI supplemented with sheep blood serum and a culture medium produced with biological material, all of these with and without supplementation of iron chelating agents. Bacterial masses were collected during the Log and Stationary phases of the bacterial growth curve, so that membrane molecules could be extracted using organic solvents. This procedure generated a fraction called membrane antigens, rich in glycoproteins, with a protein profile varying among the different growth media tested, demonstrating the importance of the medium for bacterial protein expression and consequently influencing the expressed protein profile for test production diagnoses and a future vaccine.

Keywords: *C. pseudotuberculosis.* Culture Medium. Iron Chelators. Bacterial Membrane Proteins.

Resumo

Corynebacterium pseudotuberculosis é o agente causador da linfadenite caseosa dos caprinos e ovinos, enfermidade associada a perdas econômicas diretas no setor pecuário. Além de ter um curso crônico, o seu tratamento com antibióticos é inviável e não existem vacinas eficazes disponíveis no mercado. Neste experimento, foram utilizadas condições de cultivo in vitro que se aproximassem do ambiente das condições in vivo encontradas pela bactéria durante a infecção do hospedeiro, isto incluiu a utilização de material biológico animal como meio de cultura e a restrição do ferro livre no meio. Nessas condições, era esperado que o C. pseudotuberculosis expressasse perfis proteicos de membrana ainda desconhecidos. Par isso foram utilizadas linhagens bacterianas de baixa (T1) e alta (VD57) virulência, cultivadas em caldo BHI, MQD, MQD suplementado com ferro, RPMI, RPMI suplementado com soro ovino e um meio produzido com material biológico ovino, todos estes com e sem suplementação de agentes quelantes de ferro. As massas bacterianas foram coletadas durante as fases Log e Estacionária da curva de crescimento da bactéria, para que fosse conduzida a extração das moléculas de membrana com o uso de solventes orgânicos. Este procedimento gerou uma fração denominada antígenos de membrana, rica em glicoproteínas. O perfil proteico variou de acordo com o meio utilizado, sendo que algumas bandas coradas na eletroforese aparecem em muitos meios. Portanto foi possível produzir informações importantes para uma futura vacina e produção testes diagnósticos.

Palavras-chave: *C. pseudotuberculosis. Meios de Cultura. Quelantes de Ferro. Proteínas de Membrana Bacteriana.*

1 Introduction

The caseous lymphadenitis (CL), infectious disease caused by the pathogen *Corynebacterium pseudotuberculosis* (*Cp*), is responsible for a significant degree of economic losses in sheep and goat farming. The disease has a chronic nature, being responsible for negative impacts on weight gain and milk production, as well as the leather devaluation and carcasses condemnation in small ruminant slaughterhouses. In addition to antibiotic treatment being considered unfeasible, its eradication is difficult, as it is widely disseminated in herds worldwide (DORELLA *et al.*, 2006) and so far, no satisfactory vaccine model has been produced for goats and sheeps (BASTOS *et al.*, 2012; RODRIGUES *et al*., 2018, SÁ *et al*., 2021).

Although the disease can be diagnosed through microbiological culture of caseous material, most infected animals may develop internal granulomas, commonly in the lungs or mediastinal lymph nodes, not expressing clinical signs of infection (BINNS *et al.,* 2007; LIMA *et al*., 2017). Once established in the herd, the eradication of this disease is a difficult step, due to the difficulty in treatment and the detection of animals contaminated by clinical signs has its efficiency limited (MOHAN *et al*., 2008; PAULE *et al*., 2004; SÁ *et al*., 2021). Given this complexity, new biotechnological approaches have been used by researchers to unravel the molecular mechanisms of virulence of *C. pseudotuberculosis*,

aiming to mimic *in vitro* the *in vivo* conditions in which the agent installs itself and develops the infectious picture.

Studies demonstrate that extracellular (surface) proteins play an important role in bacterial virulence, favoring mechanisms of adhesion capacity, cell invasion, adaptation, survival within the host cell, escape of defense cells, and modulate the host's immune system (HILBI; HAAS, 2012; SCHNEEWIND; MISSIAKAS, 2012).

Iron is a vital nutrient for several bacteria, however some of them have the ability to develop and multiply themselves in an iron-free environment, using other metals, such as manganese and cobalt, to perform their metabolic functions (ANDREWS *et al.*, 2003; SMITH, 2004).

Assuming that the host's infection environment has naturally low availability of iron, studied began to seek a mimicry of these conditions *in vitro* models, so that microorganisms could express the possible virulence factors that are actually expressed *in vivo*. In some studies, iron restriction for microorganisms in culture medium was achieved by supplementing with chelating agents such as deferoxamine mesylate, deferiprone and deferasirox (MAURER *et al*., 2007; PARADKAR *et al*., 2008); other studies have sought such mimicry by carrying out bacterial culture directly in human and animal serum or in industrial fetal bovine serum, which are environments naturally rich in iron chelating proteins (OOGAI *et al*., 2011). However, in cultures with Cp, the growth characteristics were not related yet, with what was expressed by bacteria in different culture conditions.

One of the most efficient ways for extracellular iron uptake found by bacteria is through the synthesis of siderophores, small biomolecules, with a size from 400 to 1000 Da, which have a very high affinity for ferric ions (CHU *et al*., 2010). Most siderophores are synthesized by non-ribosomal peptide synthases (NRPS), which are multimodular enzymes that resemble eukaryote type I fatty acid synthases (CHAN; VOGEL, 2010). This synthesis pathway was the first to be elucidated and remains the most important among bacteria, being responsible for the synthesis of many of the already known siderophores (CHU *et al*., 2010).

Iron homeostasis is regulated according to the availability of iron in the medium. Many microorganisms depend on the balance between capturing iron to grow and survive and avoid its toxic effects, this being controlled by the Fur protein (*Ferric uptake regulator*) (CROSA, 1997; HANTKE, 2001). Fur controls the expression of genes linked to the uptake and storage of this metal and genes related to: oxidative stress, energy metabolism and virulence (BENJAMIN *et al*., 2010; DA SILVA NETO *et al*., 2009; JACKSON *et al*., 2010; LITWIN; CALDERWOOD, 1993).

Although there is a growing search for diagnosis and prophylaxis for CL, little has been investigated about the molecular mechanisms of virulence in *Cp*. Two determinants of virulence have been well characterized: the exotoxin phospholipase D (*pld*) and the cell wall toxic lipids, also called corinomicolic acids. *Pld* is considered the main virulence factor of this bacterium (HODGSON *et al*., 1999), constituting a permeability factor that promotes the hydrolysis of ester bonds in the membrane sphingomyelin of mammalian cells (COYLE; LIPSKY, 1990; MCNAMARA *et al*., 1995); thus, this exotoxin contributes to the spread of the microorganism from the initial site of infection to secondary sites within the host (WILLIAMSON, 2001). On the other hand, corinomicolic acids are surface lipids of Cp that contribute to the disease pathogenesis (HARD, 1972), so that more virulent strains have more lipids than attenuated strains (JOLLY, 1966). This lipid layer provides protection against the degradative action of enzymes present in macrophage phagolysosomes, in addition to allowing microorganisms to adhere and promoting local cytotoxicity (ALVES; PINHEIRO, 1997; MUCKLE; GYLES, 1982).

Among the scientific advances generated in this area, recent studies have already registered the existence of genes and virulence factors regulated by the availability of iron, so that the gene expression profile and bacterial proteome are altered in response to iron restriction (CARPENTER *et al*., 2009; CORNELIS, 2010; JONES; WILDERMUTH, 2011). From these modifications, new virulence factors associated with iron metabolism, bacterial pathogenesis and pathogen persistence in the host were discovered in *Corynebacterium diphtheriae* (SCHMITT, 1997), *Mycobacterium tuberculosis* (CRONJÉ *et al*., 2005; RODRIGUEZ; SMITH, 2006), *Mycobacterium avium* (JANAGAMA *et al*., 2010), *Rhodococcus equi* (BOLAND; MEIJER, 2000; BARGEN *et al*., 2011), *Francisella tularensis* (LENCO *et al*., 2007) and *Chlamydophila pneumoniae* (MAURER *et al*., 2007), among other intracellular microorganisms. In the search for molecular markers that help in the early diagnosis and treatment of several diseases, many studies have focused on alterations in genes, their transcripts and protein products involved in important cellular processes (BARBOSA *et al*., 2012). The proteomes are: the products translated from genomic sequences, proteins resulting from post-transcriptional and post-translational processes, and the complexes formed by these biomolecules (AHRENS *et al*., 2010). They are dynamic and their profile changes according to physiological status and stages of cell differentiation (JENSEN, 2004).

Considering the worldwide demand for solutions for the control of caseous lymphadenitis in goats and sheep, it is necessary to carry out studies aimed at understanding the microorganism pathogenesis mechanisms and its associated molecules. In this sense, *in vitro* growth assays of this microorganism under iron-restricted conditions and in animal serum can contribute to the expression of a new protein profile, so that molecules that are fundamental to the pathogenesis and also unknown can be identified and characterized and,

subsequently, used in the development of new vaccine models and immunodiagnostics.

2 Material and Methods

In this experiment, two bacterial strains from the Collection of Microorganisms of the Institute of Health Sciences of UFBA were used. The strain called T1 was isolated from a degranuloma in a damaged caprine lymph node in the region of Santa Luz in Bahia. This strain was considered to be of low virulence for presenting synergistic hemolysis with *Rhodococcus equi* less intense than the others, as well as for not causing the death of BALB/c mice at a dose of 10^7 CFU, inducing only the development of discrete lesions. The other strain was isolated in the region of Juazeiro, state of Bahia, from goat lymph node caseous material, being cataloged as VD57. The identification was confirmed by Gram stain, colonial morphology, synergistic hemolysis with *R. equi*, urease production, catalase production and later confirmed by API *Coryne* kit (BioMérieux). Both strains mentioned, as well as the other samples of C. *pseudotuberculosis* isolated from goats' and sheep's superficial granulomas from the interior of the state of Bahia, were kept in glycerol in a freezer at –70 °C.

Five groups of cultures of the T1 strain of *C. pseudotuberculosis* were carried out to determine the growth curve under the influence of chelating agents. As a culture medium, MQD (chemically defined medium) was used in a volume of 50 ml in Falcon tubes, supplemented with 0.5% Tween 80, which enables the microorganism to grow segregated. All cultures were carried out in triplicate, kept at 37°C under agitation for 72 hours, and the experimental groups were divided as follows: G1 (control culture) inoculation in pure MQD; G2, inoculation in MQD supplemented with 50 µM FeSO4; G3, inoculation in MQD supplemented with 25 µM deferoxamine mesylate (DFX); G4, inoculation in MQD supplemented with 50 µM deferoxamine mesylate; G5, inoculation in MQD supplemented with 75 µM deferoxamine mesylate; G6 inoculation in MQD with 10 mM deferasirox; G7 inoculation in MQD with 20 mM deferasirox; G8 inoculation in MQD with 40 µM deferasirox.

Growth curve monitoring was performed by flow cytometry, as established by SAMPAIO *et al* (2019). 3.0 ml of culture was withdrawn at times 0, 2, 6, 12, 18, 24, 30, 36, 48 and 72 hours after inoculation. Bacteria were washed in 0.9% saline solution and centrifuged for 4 minutes at 8000g, for later disposal of the supernatant. SYBR Safe™ (dye that is inserted into the double strand of DNA, not depending on membrane permeability) was added, and incubated for 20 minutes in a dark room at room temperature. A new wash was performed to remove excess reagents, and the bacterial *pellet* was resuspended in a propidium iodide solution, in order to verify cell permeability. To perform the absolute count of microorganisms in suspension by the flow cytometer, *TruCount* tubes (Becton Dickson, USA) were used, containing fluorescent microspheres, with the amount of *beads* constant in each batch. After counting, the following formula was applied to the results: total bacteria = (Nº of beads events bacterial / Nº of beads A+B) X Nº of Beads per µl. SYBR Safe™

At this stage of the experiment, the lag, stationary log and decline phases in the microorganism growth curve were defined. Based on the influence exerted by chelating agents on the growth curves, the best chelator for use in the following stages of the research was defined.

The cultivation conditions applied were in accordance with the works by Lenco *et al*. (2007) (performed with *Francisella tularensis*) and Oogai *et al*. (2011) (performed with *Staphylococcus aureus*), suffering only minor adaptations. Lines T1 (low virulence) and VD57 (high virulence) of *C. pseudotuberculosis* were cultivated in 250 ml of BHI broth, Fetal Bovine Serum (SFB), MQD (composition Table 1), MQD supplemented with iron, RPMI (composition Table 2), RPMI supplemented with sheep serum and medium produced from sheep biological material, all under agitation (200 rpm) in automatic *shaker* for 48 hours; the means were used intact (rich in iron) or supplemented with the chelating agent defined in the previous step, for the respective establishment of an iron-restricted environment.

Table 1 - MQD composition in g/L

Phosphate-buffered saline:0.067 M		**Aminoacids**	
	Na2HPO4 72%	Arginine	0.2528
Na2HPO4 (7H2O)	12.93 g	Cysteine	0.0480
KH2PO4 (anidro)	2.55 g	Histidine	0.0840
NH4Cl	1.00 g	Isoleucine	0.1050
CaCl2	0.02 g	Leucine	0.1048
MgSO4	0.20 g	Lysine	0.1450
Glucose	12.00 g	Methionine	0.0302
Tween 80	1.00 g	Phenylalanine	0.0660
		Threonine	0.0952
Vitamins		Tryptophan	0.0204
Calcium Pantothenate	0.0010	Tyrosine	0.0720
Choline Chloride	0.0010	Valine	0.0936
Folic Acid	0.0010	Alanine	0.0356
Inositol	0.0020	Asparagine	0.0528
Niacinamide	0.0020	Aspartic Acid	0.0532
Pyridoxine	0.0010	Glutamic Acid	0.0532
Riboflavin	0.0001	Glycine	0.0300
Thiamine HCl	0.0010	Serine	0.0420

Source: Research data.

Table 2 - RPMI composition

Inorganic Salts	**mg/L**	L-tryptophan	5
Ca(NO3)2.4H2O	100	L-prolina	20
KCL	400	L-tyrosine	28
MgSO4.7H2O	100	L-serine	30
NaCl	6.000,00	L-valine	20
NaHCO3	2.000,00	L-threonine	20
Na2HPO4	800		
		Vitamins	**mg/L**
Aminoacids	**mg/L**	Biotin	0.2

L-arginine.HCL	200	Calcium Pantothenate	0.25
L-asparagine. H2O	50	Choline Chloride	3
L-aspartic acid	20	Folic Acid	1
L-cysteine	50	Inositol	35
L-glutamic acid	20	Niacinamide	1
L-glutamine	300	p-aminobenzoic acid	1
Glycine	10	Pyridoxine.HCL	1
L-histidine.HCL. H2O	15	Riboflavin	0.2
L-hydroxyproline	20	Thiamine.HCL	1
L-isoleucine	50	B-12 vitamin	0.005
L-leucine	50		
L-lysine.HCL	40	**Other Components**	**mg/L**
L-methionine	15	Glucose	2.000.00
L-phenylalanine	15	Glutathione	1
		Phenol red	5

Source: Research data.

Cultivations were carried out in duplicates; a first replicate was removed from the *shaker* during the intermediate period of the Log growth phase (defined in the previous step); the second replicate was removed from the *shaker* in the intermediate period of the Stationary phase (defined in the previous step). The media were centrifuged for 40 minutes at 4000g at 4 ºC to remove the supernatant from the cultures. Subsequently, the bacterial pellets were washed 3 times in PBS Tween 0.05%.

Table 3 - Experimental groups

Growth Medium	Group	Chelating Agent	Cepa Cp
RPMI	25	-	-
	26	-	-
	27	-	VD 57
	28	-	VD 57
	29	-	T1
	30	-	T1
	31	DFX* 25 µmol	VD 57
	32	DFX* 25 µmol	VD 57
	33	DFX* 25 µmol	T1
	34	DFX* -25 µmol	T1
BHI	35	-	-
	36	-	-
	37	-	VD 57
	38	-	VD 57
	39	-	T1
	40	-	T1
	41	DFX* 25 µmol	VD 57
	42	DFX* 25 µmol	VD 57
	43	DFX* 25 µmol	T1
	44	DFX* 25 µmol	T1
MQD + IRON	45	-	-
	46	-	-
	47	-	VD 57
	48	-	VD 57
	49	DFX* 25 µmol	VD 57
MQD + IRON	50	DFX* 25 µmol	VD 57
	51	DFX* 50 µmol	VD 57
	52	DFX* 50 µmol	VD 57
	53	DFX* 75 µmol	VD 57
	54	DFX* 75 µmol	VD 57
	55	-	T1
	56	-	T1
	57	DFX* 25 µmol	T1
	58	DFX* 25 µmol	T1
	59	DFX* 50 µmol	T1
	60	DFX* 50 µmol	T1
	61	DFX* 75 µmol	T1
	62	DFX* 75 µmol	T1
RPMI + IRON	63	-	-
	64	-	-
	65	-	VD 57
	66	-	VD 57
	67	DFX* 25 µmol	VD 57
	68	DFX* 25 µmol	VD 57
	69	-	T1
	70	-	T1
	71	DFX* 25 µmol	T1
	72	DFX* 25 µmol	T1
RPMI + Blood Serum	73	-	-
	74	-	-
	75	-	VD 57
	76	-	VD 57
	77	DFX* 25 µmol	VD 57
	78	DFX* 25 µmol	VD 57
	79	-	T1
	80	-	T1
	81	DFX* 25 µmol	T1
	82	DFX* 25 µmol	T1
MQD	83	-	-
	84	-	-
	85	-	VD 57
	86	-	VD 57
	87	DFX* 25 µmol	VD 57
	88	DFX* 25 µmol	VD 57
Adultal	89	-	-
	90	-	-
	91	-	VD 57
	92	-	VD 57
	93	DFX* 25 µmol	VD 57
	94	DFX* 25 µmol	VD 57
Fetal Bovine Blood Serum	95	-	-
	96	-	-
	97	-	VD 57
	98	-	VD 57
	99	DFX* 25 µmol	VD 57
	100	DFX* 25 µmol	VD 57
	101	-	T1
	102	-	T1
	103	DFX* 25 µmol	T1
	104	DFX* 25 µmol	T1

*Deferoxamine Mesylate (DFX)

Source: Research data.

The fractions obtained were submitted to the electrophoresis procedure in a 15% polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE); later, the gels were stained using silver nitrate or electro transferred to a nitrocellulose membrane for *Western Blot*. After transfer, the membrane was blocked with PBS-T plus 5% bovine serum albumin for 12 hours at 4 °C; then, it was incubated at 37 °C for 1 h with a pool of sheep serum samples with confirmed infection by *C. pseudotuberculosis* diluted 1:50 in PBS-T containing 1% skim milk powder. A pool of sera from animals confirmed to be negative for *C. pseudotuberculosis* infection was used as a negative control for the tests.

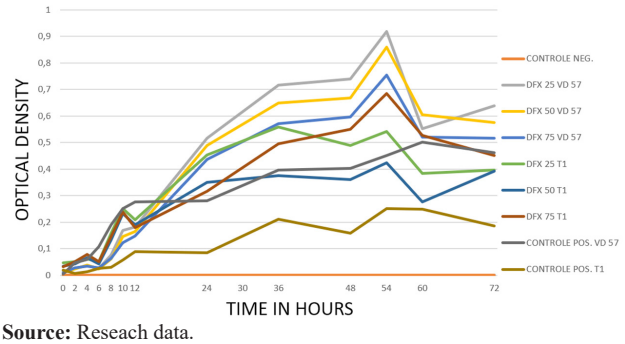
3 Results and Discussion

In infections, the host does not easily release iron to microorganisms (ANDREWS *et al*., 2003; ANDREWS, 2010; HOOD; SKAAR, 2012; GANZ; NEMETH, 2015). To mimic this condition using iron chelators in different culture media, it was possible to observe differences in bacterial growth curves and protein expression observed in the SDS-PAGE.

First, it was standardized which concentrations of chelators would be used, as described in the methodology for groups G1 to G7. At all points of collection of the growth curve, a GRAM-stained smear was made to assess the morphotintorial characteristics, where we no possible contamination were observed.

Deferasirox (DFS - C25H48N6O8) has an excipient that makes the culture medium cloudy, which made it impossible to read in the optical densitometer, so it was not possible to use it in the experiment. In Figure 1, it is possible to observe that in cultures with a lower concentration of chelator the bacteria grew more, as the concentration increases, bacterial growth decreased, however the chelator favored bacterial growth when compared to the culture medium only with the strain (CONTROL POS.), possibly because it has a large amount of carbon in its composition.

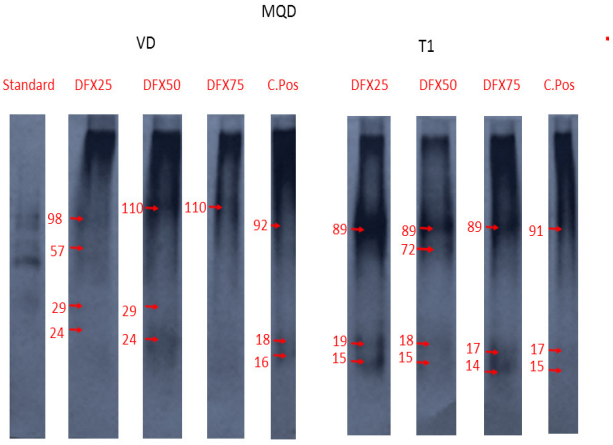
Figure 1 - Growth curve testing different concentrations of the chelator Deferroxamine Mesylate (DFX), in chemically defined medium (MQD), against the two strains of Cp tested, the NEG CONTROL only has the culture medium and the POS CONTROL the culture medium with the bacterial strain



Source: Reseach data.

In Figure 1, it is possible to observe that in cultures with a lower chelator concentration the bacteria grew more, as the concentration increases, the bacterial growth decreased, however the chelator favored bacterial growth when acquired in the culture medium only with the strain (CONTROL POS.). With the SDS PAGE, the protein profile of all cultures was analyzed, in Figure 2, although the chelator at lower concentrations favored microbial growth, no difference was observed in the protein profile in the different groups.

Figure 2 - SDS PAGE 12.5%, protein profile of the culture of VD57 and T1 strains of Cp, in MQD testing different concentrations of chelator concentrations, the numbers above of each run represent the group according to Table 1 and the numbers in red the molecular weight of the bands that appeared on silver nitrate staining

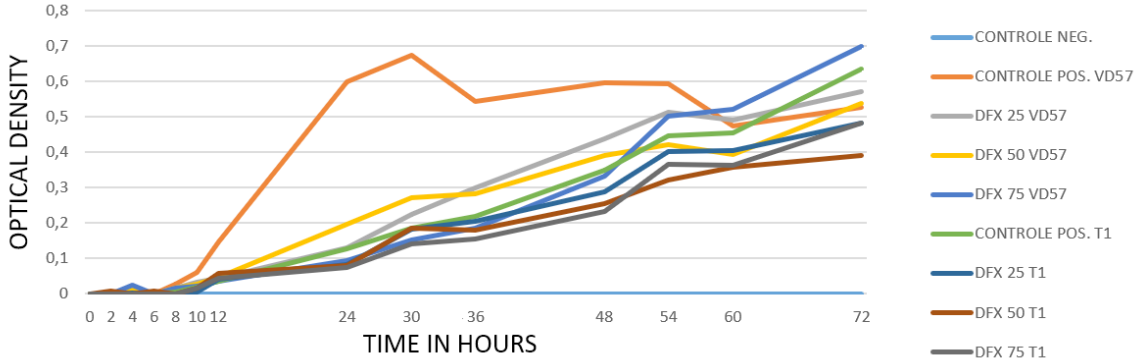


Legend: Deferoxamine Mesylate (DFX), Positive Control (C.Pos) - DFX-free medium

Source: Reseach data.

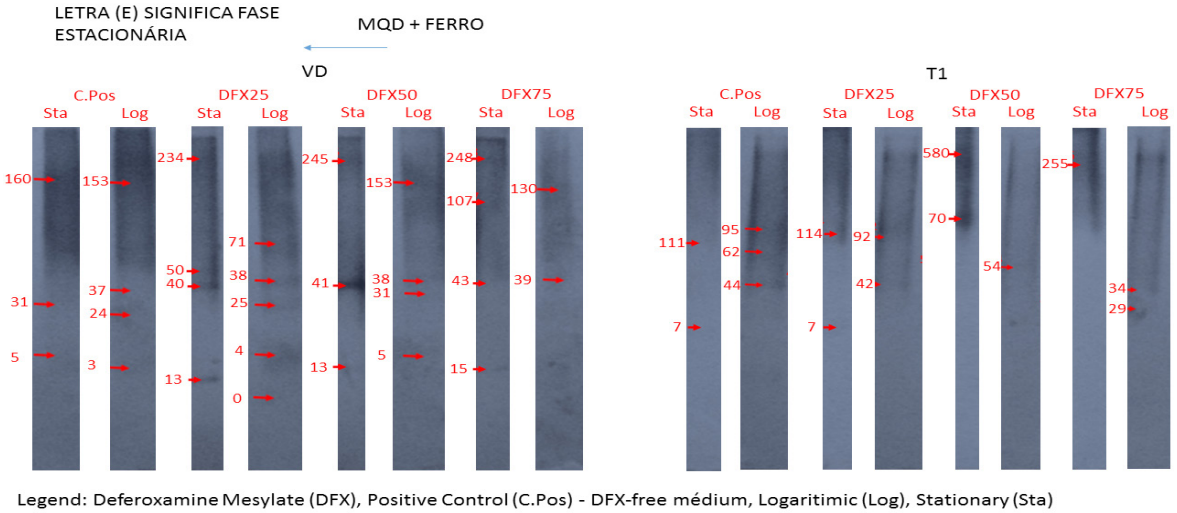
Growth curves were made with the different culture media, with and without the use of DFX and SDS PAGE electrophoresis from all the groups, some of which collected material in the logarithmic and stationary phases. To better assess the influence of iron on Cp, the MDQ was supplemented with 3.15g/L of iron chloride hexahydrate, groups 45 to 62, it was observed that the chelator caused the phase. log was longer (Figure 3), justified by the use of the chelator that mimics the *in vivo* condition, where it captures the iron in the medium through a possible pathway that is the synthesis of siderophores (CHU *et al*., 2010). The log phase is longer because the bacterium has less available iron, it is possible that it expresses more siderophores under these conditions, where it was observed that the presence of iron stimulated growth with the chelator. As well as the VD 57 strain, it has a greater capacity to capture iron. In the MQD electrophoresis supplemented with iron Figure 4, it was possible to observe that a few more bands were stained when compared to Figure 2, and that the protein profile of the stationary and logarithmic phases differ in some bands (RAMAN *et al*., 2008; HU; COATES, 2001; MAGNUSSON *et al*., 2005; DAHL *et al*., 2003).

Figure 3 - Growth curve testing different concentrations of the chelator Deferroxamine Mesylate (DFX), in chemically defined medium (MQD) supplemented with iron chloride hexahydrate, against the two strains of Cp tested, the NEG. The CONTROL only has the culture medium and the POS. CONTROL - The culture medium with the bacterial strain



Source: Reseach data.

Figure 4 - 12.5% SDS PAGE, protein profile of the culture of VD57 and T1 strains of Cp, in MQD supplemented with Iron, testing different concentrations of chelator, numbers above each run represent the group according to Table 1, the with the letter E are the samples collected in the stationary phase and the other in the logarithmic phase and the numbers in red are the molecular weight of the bands that appeared in the silver nitrate staining



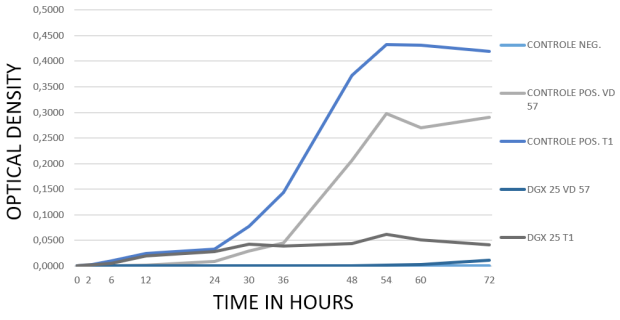
Legend: Deferoxamine Mesylate (DFX), Positive Control (C.Pos) - DFX-free médium, Logaritimic (Log), Stationary (Sta)

Source: Reseach data.

When in large concentrations, Iron can affect the microbial digestion process (CHEN *et al.,* 2008), possibly this is a factor that acts in media with higher proportions of the metal.

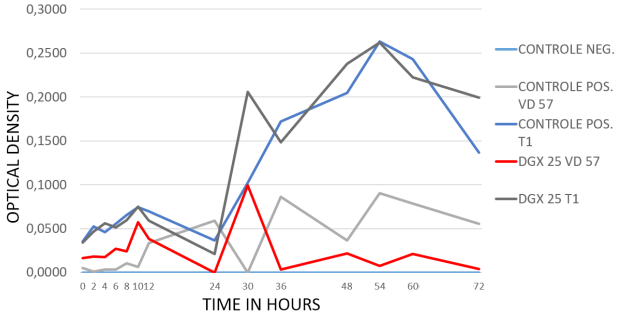
In the RPMI medium supplemented with iron chloride hexahydrate, the strains did not grow. However, when RPMI was supplemented with sheep serum, it was observed that the chelator drastically inhibited the bacterial growth, Figure 5, the same occurred with the non-supplemented medium, Figure 6.

Figure 5 - Growth curve testing RPMI supplemented with sheep serum associated with the chelator, against the two strains of Cp tested, the NEG CONTROL only has the culture medium and the POS CONTROL. the culture medium with the bacterial strain



Source: Reseach data.

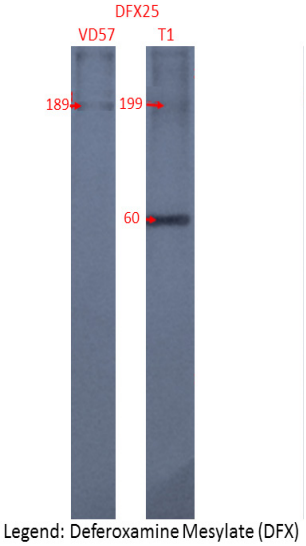
Figure 6 - Growth curve testing RPMI associated with the chelator, against the two strains of Cp tested, the NEG CONTROL only has the culture medium and the POS CONTROL, the culture medium with the bacterial strain



Source: Reseach data.

In the gels produced from bacterial masses produced in RPMI medium associated with the chelator, there was not enough bacteria mass production to make it possible to run the polyacrylamide gel, in Figure 7 (RPMI supplemented with sheep serum) only sample 28 produced mass sufficient, where the dosage of protein allowed the performance of electrophoresis.

Figure 7 - SDS PAGE 12.5%, protein profile of the culture of VD57 and T1 strains of Cp, in RPMI supplemented with sheep serum, the numbers above of each run represent the group according to Table 1 and the numbers in red the weight of the bands that appeared on silver nitrate staining

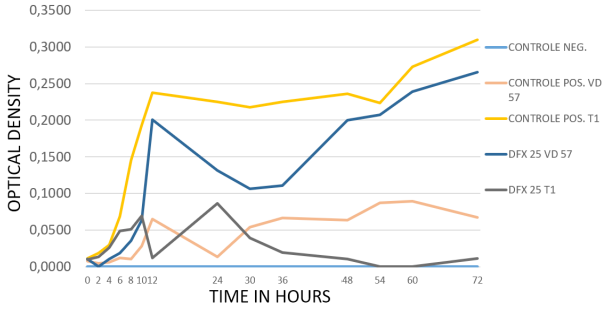


Legend: Deferoxamine Mesylate (DFX)

Source: Reseach data.

Testing the most used culture medium in this research line (BHI), associated with the chelator, it is possible to see in Figure 8 that the T1 strain, when in contact with DFX, could not develop, but VD 57 had a greater growth than the control group, and no difference was observed in the protein profile when compared to the work published by Raynal *et* al. (2018), Flores *et al.* (2020).

Figure 8 - Growth curve testing BHI associated with the chelator, against the two strains of Cp tested, the NEG CONTROL only has the culture medium and the POS CONTROL, the culture medium with the bacterial strain

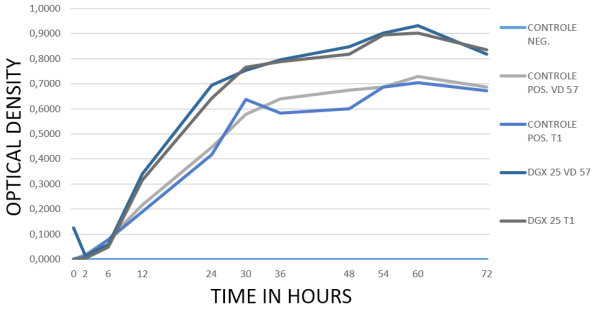


Source: Reseach data.

Possibly the T1 strain was not inoculated in the adequate amount, and it is necessary to repeat this curve to confirm the result, which is expected that it will have the same growth profile as the VD57 strain.

Seeking to approach the animal model, fetal bovine serum has been used in order to mimic the growth conditions that Cp would have in sheep and goats. Thus, the same tests were performed with the chelator (Figure 9), where an increase was observed in optical density, which is directly related to the increase in the number of microorganisms in the culture medium (SAMPAIO *et al*, 2019).

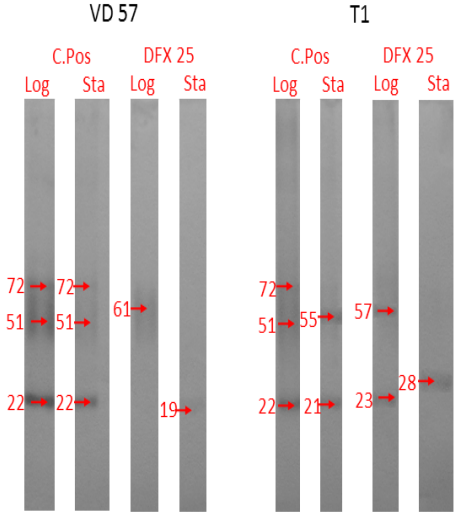
Figure 9 - Growth curve testing FBS associated with the chelator, against the two strains of Cp tested, the NEG CONTROL only has the culture medium and the POS CONTROL, the culture medium with the bacterial strain



Source: Reseach data.

In the FBS groups that had the chelator in their composition, they obtained a protein concentration much lower than the FBS groups without the chelator (Figure 10), although the bacterial growth was higher.

Figure 10 - SDS PAGE 12.5%, protein profile from the cultivation of VD57 and T1 strains of Cp, in the FBS, numbers above of each run represent the group according to Table 1, those with the letter E are the samples collected in the stationary phase and the other in the logarithmic phase and the numbers in red the molecular weight of the bands that appeared in the silver nitrate staining



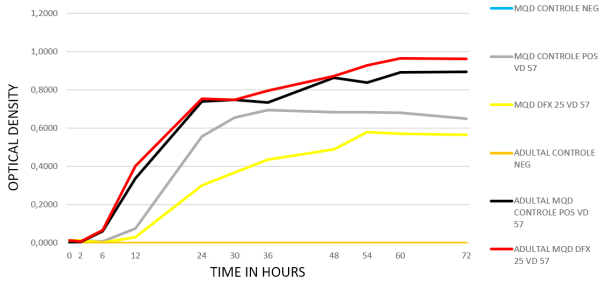
Legend: Deferoxamine Mesylate (DFX), Positive Control (C.Pos)

Source: Reseach data.

Once the results were obtained from FBS culture, the chelator was tested in a growth medium produced at LABIMUNO (Laboratory of Cellular and Molecular Immunology/UFBA), which was named ADULTAL; it was

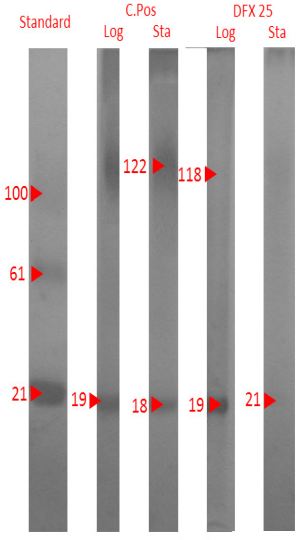
produced from sheep blood serum that had no clinical signs of deseases as well as negative sorological tests for caseous lymphadenitis. Strain VD 57 was cultivated in MQD in parallel (Figure 11). It was clear that VD 57 grew more in the culture medium called ADULTAL (groups 84 to 104). Possibly due to its richness in nutrients, as well as the Lag phase was much shorter than in MQD, the Log phase had a faster growth peak and the point after 72 hours of cultivation which would be the stationary phase continued to grow while that of the MQD was already in decline. In electrophoresis, the medium without chelator did not present the 122 KDa band of the run in the Log phase collection and 118KDa in the Stationary phase run (Figure 12), possibly the chelator has inhibited the expression.

Figure 11 - Growth curve testing ADULTAL culture medium, and MQD as a comparative model against VD57 Cp strain, CONTROL NEG only has the culture medium and CONTROL POS, the culture medium with the bacterial strain



Source: Reseach data.

Figure 12 - 12.5% SDS PAGE, protein profile of Cp VD57 strain culture in ADULTAL medium, the numbers above of each run represent the group according to Table 1, those with the letter E are the collected samples in the stationary phase and the other in the logarithmic phase and the numbers in red the molecular weight of the bands that appeared in the staining with silver nitrate



Legend: Deferoxamine Mesylate (DFX), Positive Control (C.Pos)
Source: Reseach data.

With these results, it was proved the importance of the iron chelator in the Cp growth rate. The ADULTAL medium possibly has natural chelators so it presented similar growth kinetics as the same medium associated with the chelator.

According to research by Ellis *et al*. (1991a), Ellis *et al.* (1991b), Braithwaite *et al*. (1993), Sting *et al.* (1998) and Paule *et al*. (2004b), the molecules detected in this experiment had already been identified by them (Table 4), it was tried to work with some proteins: PknG (Protein kinase G, 83KDa), SodC (Superoxide dismutase C, 21KDa) and NanH (Neuraminidase H, 74.6KDa), SpaC (Adesin, 86KDa) (SANTANA JORGE *et al*., 2016, CORREA *et al.*, 2018), where it was observed that their molecular weights match those found in this work, where it is possible to consider their presence in the electrophoresis in this study. Proteins that are already known as PLD and Catalase A appeared frequently in electrophoresis (RAYNAL *et* al., 2018; FLORES *et al.*,2020).

Table 4 - Molecules presented in the literature regarding *C. pseudotuberculosis*

Reference	Antigens				
	> 80 kDa	**80 – 60 kDa**	**59 – 40 kDa**	**39 – 20 kDa**	**< 20 kDa**
Ellis *et al.* (1991a)	100	79; 63	56; 45.7	39.8; 35.5; 31.6; 22; 20	----
Ellis *et al.* (1991b)	120; 100	79.4; 75; 70; 63	----	39.8; 36.3; 31.6; 25.1	12
Braithwaite *et al.* (1993)	120; 84	64	58; 48	36; 33; 30	14
Muckle *et al.* (1992)	120	68; 64	43; 40	31.5; 22	-----
Sting *et al.* (1998)	110	80; 66	55; 48; 45	30	-----
Paule *et al.* (2004b)	125; 108; 90; 85	75; 72; 68; 64	53; 50; 43; 41; 40	31; 28; 24	-----

Source: Reseach data.

4 Conclusion

Considering the importance of sheep and goat farming products to the economic outlook and the lack of research about *Corynebacterium pseudotuberculosis* related to Caseous Lymphadenitis, it was clear that information was produced aiming to fill some existing gaps regarding the microorganism. Therefore, the results found corroborate the science, as they will serve as a foundation for further research, through the visualization of possible virulent molecules in conditions that closely resemble *in vivo* conditions.

According to the experimental protocol developed and the results obtained, it is concluded that: different growth culture conditions result in different induction of these molecules, proteins appeared in electrophoresis that are already well known as PLD and Catalase A, and a few others not identified yet and the production of growth culture that simulated the conditions found by bacteria in the animal presented growth and protein expression characteristics different from other culture media .

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