Production of outer membrane vesicles by the human pathogen *Burkholderia cenocepacia*: purification and preliminary characterization

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Abstract

Cystic Fibrosis (CF) is an autossomal recessive disease resulting from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, leaving affected patients more susceptible to infection by bacteria. One group of bacteria, the *Burkholderia cepacia* complex (Bcc), is responsible for dangerous infections in these patients, due to their resistance to multiple antibiotics being therefore necessary to develop alternative treatments. Outer membrane vesicles (OMV) have been used for the development of vaccines against pathogens such as *Neisseria meningitidis* and *Burkholderia pseudomallei*, with an OMV vaccine against *N. meningitidis* already available in the market. This study aimed at the development of a scalable production method of OMVs, in order to allow their use in vaccine development studies. OMVs from *B. cenocepacia* K56-2 were isolated and purified by size exclusion chromatography (SEC), however the final purity was not high, with OMVs still contaminated with pili and flagella. This prompted the development of an alternative strategy to obtain purified OMVs in which the strain used for OMV production was changed to *B. cenocepacia* AU1054 non-piliated strain. With this strain it was possible to obtain contaminant-free OMVs after the isolation process, which was confirmed by transmission electron microscopy (TEM). OMVs obtained from *B. cenocepacia* AU1054 were used to perform cytotoxic assays in 16HBE14o- human bronchial epithelial cells and in *Galleria melonella*. 16HBE14o- cells suffered reduced survival upon exposure to AU1054-derived OMVs, however this result was not reproduced in *G. melonella,* with all injected caterpillars surviving the assay.

Keywords: Outer membrane vesicles, Cystic fibrosis, purification, *Burkholderia cepacia* complex, Size exclusion chromatography, cytotoxicity

Resumo

A Fibrose quística (FQ) é uma doença autossómica recessiva resultante de mutações no gene responsável pelo regulador de condutância transmembranar de FQ, levando a que estes pacientes fiquem suscetíveis a infeções bacterianas. Um grupo de bactérias, complexo de *Burkholderia cepacia* (Bcc), é responsável por perigosas infeções em pacientes com FQ, devido à elevada resistência a antibióticos que estas bactérias exibem sendo necessário desenvolver estratégias alternativas para evitar estas infecções. As vesículas da membrana exterior (VME) têm sido utilizadas para o desenvolvimento de vacinas contra bactérias tais como *Neisseria meningitidis* e *Burkholderia pseudomallei*, estando já disponível no mercado uma vacina contra *N. meningitidis*. Neste estudo pretendia-se desenvolver um método escalável para a purificação de VME. Estas foram isoladas de *B. cenocepacia* K56-2 e submetidas a um passo purificação por cromatografia de exclusão molecular (CEM). A purificação de VME não atingiu os resultados desejados, pelo que foi desenvolvida uma estratégia alternativa que consistiu no uso de uma estirpe de *B. cenocepacia* AU1054. Com esta nova estirpe foi possível obter VME livres de contaminantes após o isolamento das vesículas, como confirmado por microscopia de transmissão electrónica (TEM). Estas VME foram posteriormente utilizadas em ensaios citotóxicos, realizados em células do epitélio bronquial humano 16HBE14o- e em larvas de *Galleria melonella.* As células 16HBE14o- sofreram uma redução na sobrevivência celular após exposição a VME derivadas de *B. cenocepacia* AU1054, no entanto este resultado não foi confirmado nos ensaios realizados com *Galleria melonella*, em que todas as lagartas injectadas sobreviveram ao ensaio.

Palavras-chave: Vesículas da membrana exterior, Fibrose quística, purificação, Complexo de *Burkholderia cepacia*, cromatografia de exclusão molecular, citotoxicidade.

Table of contents

Index of Figures

Index of Tables

List of abbreviations

2-DE- 2-Dimensional gel electrophoresis

BCA- Bincinchoninic acid

BSA- Bovine albumin serum

Bcc- *Burkholderia cepacia* complex

CF- Cystic fibrosis

CFTR- Cystic Fibrosis transmembrane conductance regulator

EPS- Exopolysaccharides

FBS- Fetal bovine serum

IEF- Isoelectric focusing

IPG- Immobilized pH gradient technique

LB- Luria-Bertani

LPS- Lipopolysaccharide

MALDI- Matrix-assisted laser desorption ionization

MEM- Minimum essential medium with Earle's salt

MS- Mass spectrometry

MTT- [3-(4,5 dimethylthiazol-2-yl-2,5 tetrazolium bromide)]

NMWC- Nominal molecular weight cut-off

OD_{640nm}- Optical density at 640nm

OMP- Outer membrane proteins

OMV- Outer membrane vesicles

PBS- Phosphate buffered saline

PQS- *Pseudomonas* Quinilone signal

SDS-PAGE- Dodecyl sulfate polyacrylamide gel

SEC- Size Exclusion Chromatography

TAA- Trimeric autotransporter adhesions

TEM- Transmission electron microscopy

TOF- Tandem time of flight

1. Introduction

1.1 Cystic fibrosis

Cystic fibrosis (CF) is an autossomal recessive disease resulting from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes for a cyclic-AMP-regulated chlorine channel, causing defective transport of chlorine and hydrogen carbonate in epithelial cells [1; 2]. The consequences of this defect are complex, but the impairment in ion transport across epithelial surface will affect the mucus characteristics and cause mucus build-up [3], increasing the possibility of bacterial infections [4].

This disease affects several organs, such as the lungs, pancreas, the gastrointestinal tract, among other organs [1]. The main cause of death in CF patients is lung failure, which may be caused by recurrent or chronic infection [5].

The main bacteria responsible for the infections caused in the CF patients are *Staphylococcus aureus*, for younger patients (less than 18 years old), and *Pseudomonas aeruginosa*, for older patients. Other pathogens infecting CF patients include *Haemophilus influenzae, Achromobacter xylosoxidans, Stenotrophomonas maltophilia, Burkholderia cepacia* complex (Bcc), as well as multi resistant forms of *P. aeruginosa* and *Staphylococcus aureus*. Bcc bacteria are responsible for a small percentage of the total CF infections cases, with only approximately 5% of occurrences. However these bacteria are well known for their capability of surviving a wide array of the most frequently used treatments in CF patients, thus becoming extremely difficult to eradicate [6]. In a subset of CF patients, lung infections with these bacteria lead to declining lung function, with necrotizing pneumonia and a rapidly fatal septicaemia termed "*cepacia* syndrome" [7]. This syndrome is not found in CF patients colonized by any other common CF pathogens.

1.2 Burkholderia cepacia complex

Bcc is a group of closely related bacterial species which are ubiquitous in nature [8]. Bcc is currently composed by 20 species, however this number may increase since a large number of candidate species awaits formal naming [9]. Despite all Bcc species had strains recovered from CF patients the most common ones within CF infections are *Burkholderia cepacia, Burkholderia cenocepacia, Burkholderia multivorans*, *Burkholderia dolosa, Burkholderia stabilis* and *Burkholderia vietnamensis* [6]. The virulence and transmissibility of each strain is variable with *B. cenocepacia* and *B. multivorans* being the most problematic, together they account for 85% to 95% of CF infections [6]. They have the capability to disseminate and persist among CF patients, are exceptionally resistant to many antimicrobial agents, and once acquired are rarely eradicated [10]. They are also responsible for several outbreaks and frequently associated with highest risk of developing fatal "*cepacia* syndrome" [11]. The infection with these bacteria, especially with *B. cenocepacia*, poses a problem for lung

transplants, which are common for CF patients, since it increases the mortality of the patients when infected with *B. cenocepacia* before the transplant [12].

Bcc bacteria may also be found in the soil, where it has an important role in certain crops, in which they develop beneficial functions for the plant due to nitrogen fixation [13]. However the existence of these bacteria in the soil is not the main route of acquisition, the transmission and acquisition of these infections is mainly through patient contact and hospital units, where there are reports of this bacteria being found in contaminated disinfectant solutions, saline solutions and medical instruments [14; 15; 16].

Infection with Bcc bacteria is not exclusive of CF patients, there are several reports of Bcc infections in non-CF patients [17], particularly in chronic granulomatous disease patients, immunocompromised patients, such as oncologic patients [18], diabetic patients [19], patients in intensive care units [20] and haemodialysis patients [21].

1.2.1 Bcc virulence factors

As mentioned before, Bcc bacteria exhibit several characteristics that difficult the most commonly used treatments for Bcc-infected patients, specially due to their high resistance to multiple antibiotics [22; 10], the ability to form biofilms [23], the capacity to adhere and invade respiratory epithelial cells, and evasion of host immune system [24].

Although pathogenesis of Bcc infections is not fully understood, several virulence determinants have been described and multiple factors contribute to bacterial virulence. *B. cenocepacia* is the most representative and well characterized Bcc species. Many virulence determinants are proposed to play a role in the ability of *B. cenocepacia* to cause disease.

The *Quorum sensing* mechanism is an important virulence mechanism it controls the expression of several genes, some of them are important for virulence [25]. The impairment of *cep* quorum-sensing system in *B. cepacia* and *B. cenocepacia* results in reduced virulence in infection models, possibly due to the regulation of a zinc metalloprotease (*zmp*A) by this system [31]. Another important mechanism which is regulated by *quorum sensing,* and with an important role in virulence, is the formation of biofilms [27]. The capability of forming biofilms is an important virulence determinant since bacteria are more resistant to antibiotics when in biofilms than in planktonic conditions [29]. Bcc bacteria are resistant to a wide range of antibiotics which difficult the clearance of infection [22; 10]. Besides the capability of resisting to antibiotics it is also important for infecting bacteria to uptake as much iron as possible. The capability of uptaking iron is an important characteristic during infection, due to its reduced availability. *B. cenocepacia* has the ability of producing Ornibactins and Siderophores to facilitate the uptake of iron, and mutants without the capability of producing these proteins exhibit reduced virulence [30,31].

Exopolysaccharides (EPS) are not present in all Bcc strain, however when present facilitate the development of a persistent infection [32]. The presence of EPS is strain-dependent, it increases the persistence when present however the absence of EPS does not rule out the possibility of developing severe infections, thus the presence of EPS is a determinant of virulence, with strains possessing EPS showing increased virulence [10].

The capability of bacteria to be mobile and to adhere to host's cells are important characteristics for the development of infection. The presence of flagella influences the motility of the bacteria, and when impaired difficult the invasion of hosts cells, however this reduction in invasion occurs not due to reduced adherence but due to the reduced motility caused by the impairment in flagella [33]. Regarding the adherence of bacteria, different proteins are involved in the process. Cable pilus mediate cell-to-cell interactions and plays a role in adherence to both cellular and acellular surfaces [34]. Another group of proteins responsible for adherence are trimeric autotransporter adhesins (TAAs), these proteins intervene in biofilm formation, adherence and invasion to hosts cells and virulence in *Galleria mellonella* host [35].

All of these virulence factors combined affect the virulence of the bacteria and may cause more or less severe infections depending on their presence or absence. In general these factors are commonly observed in Bcc species, thus justifying the problems associated with these infections.

1.3 Strategies to combat bacterial infections in CF patients

Currently the main strategies to combat bacterial infection in CF patients rely on the use of antibiotic therapies. These antibiotic therapies are targeted to the principal bacteria identified on the infected CF patients [37;38]. For treatment against Bcc infections antibiotics such as doxycicline, orally administered, and ceftazidine, intravenously administered, among others, are recommended [38]. Some antibiotics may be combined, increasing bactericidal capability, and used to combat CF bacterial infections [39]. The use of tircacillin conjugated with clavulanic acid for treatment of Bcc infections and fosfomycin conjugated with tobramycin for treatment of *P. aeruginosa* infections, are examples of antibiotics combinations for the treatment of bacterial infections in CF patients [38].

An alternative strategy to the intravenous and oral administration of antibiotics, is the administration of antibiotics by aerosols, such as tobramycin [40] and aztreonam [41]. The administration of antibiotics by inhalation achieves high pulmonary concentrations and lowers systemic side effects. However, pulmonary deposition of inhaled drugs is substantially limited by bronchial obstruction with viscous mucus and restrained by intrapulmonary bacterial biofilms [42; 43].

One strategy to increase the efficiency of antibiotics is the encapsulation of antimicrobial peptides in liposomes. This process increases the efficiency of the antimicrobial peptides in combating the infection, and may have great potential for use in CF therapy [42].

The antibiotic resistance and the ability to acquire resistance during treatment coupled with the inefficiency of current therapies to fight Bcc chronic infections in CF patients, present an increasing urge to develop new strategies to prevent Bcc infections.

1.3.1 Vaccine development

There have been several attempts to develop a vaccine against the Bcc species and different approaches have been evaluated. These strategies consisted in the use of protein subunits, such as lipopolysaccharides (LPS) core antigen, exopolysacharides, flagella antigen, metalloproteases, among

others [44; 45]. One of the most promising targets for vaccine development against Bcc bacteria is the use of outer membrane proteins (OMPs). Studies performed concluded that intranasal immunization with *B. cenocepacia* OMPs in mice increased clearance of *B. cenocepacia* and may provide cross-protection against *B. multivorans* [46].

1.4 Outer membrane vesicles

Outer membrane vesicles (OMVs) are constitutively blended from the outer membrane of bacterial cells and in the last decade have played an important role in vaccine development. These vesicles present several characteristics desirable for this purpose, they are acellular, carry multiple immunogenic components as the pathogen and are stable when stored at 4ºC [47; 48]. These characteristics make OMVs a suitable candidate for vaccine development.

1.4.1 OMVs as a vaccine candidate

Recently there has been an increasing interest in vaccine development using OMVs, with studies performed in several Gram-negative bacterial species. Studies have been performed in *Helicobacter pylori* [49]*, P. aeruginosa* [50]*, Neisseria meningitidis* [51] and *Burkholderia pseudomallei* [52] demonstrating that OMVs have the ability of activating pro inflamatory responses.

The inflammatory response is activated by several proteins in the membrane of the vesicles, such as adhesins from *N. meningitidis* (NadA) [53] and porins [54]. These proteins lead to increased macrophage and dendritic cells response activation, eventually leading to the stimulation of memory cells [55], providing long-term resistance. Besides that, the differences in the composition of OMVs compared with the bacterial outer membrane [50; 56], may provide a pathogen specific response than the stimulation by purified LPS or OMP [47].

Regarding the development of OMVs-based vaccines, several studies have been performed and there is already a OMV vaccine in the market against *N. meningitidis* B (Bexsero®), this vaccine uses vesicles from an epidemic strain from New Zealand [57]. Development of a vaccine against *B. pseudomallei* has also demonstrated promising results, providing significant protection to mice against lethal challenges with *B. pseudomallei* [52; 58]; and activating humural immuneresponses, without associated toxicity, in Nonhuman primates upon exposure to *B. pseudomallei* OMVs [59]. Studies approaching the development of vaccines against other bacteria have also been developed, more precisely regarding *Salmonella enterica* Typhimuriun [60], *Bacillus anthracis* [61], *Vibrio cholerae* [62] and *Bordetella pertussis* [63].

1.4.2 OMVs characterization

As said before OMVs are produced from the outer membrane of Gram-negative bacteria, these vesicles do not result from membrane instability, cell death or cell lysis, and are formed during all growth phases as the outer membrane blebs outward and pinches off [64; 65; 66]. OMVs are spherical

lipid bilayer proteoliposomes, with sizes ranging from 20 to 300nm, they are associated with outer membrane proteins, lipopolysaccharides and other proteins related with virulence [65; 67; 68]. Blended vesicles are produced by organisms from the three branches of the tree of life. They are produced by Gram-negative and Gram-positive bacteria [61; 69], fungi [70], archaea [71], and even in parasites [72]. Despite some small differences in the production of the vesicles this seems to be an evolutionary conserved mechanism [73].

There are different mechanisms suggested for the formation of OMVs. One of the proposed mechanisms is the temporary local disruption of links between outer membrane of the cell and the proteoglycan layer. The links between these two layers are regulated by proteins which are anchored to both layers, such as OmpA and the complex formed by the proteins Pal, TolB and TolA. The presence of these proteins increases the stability of the membrane [74; 75], and OMVs are formed in areas where the presence of these proteins is reduced, thus reducing the cross-links between outer membrane and the proteoglycan layer, facilitating the formation of vesicles [76].

Another suggested model for the formation of OMVs is the accumulation of over-expressed periplasmic proteins, which induces the formation of vesicles by causing an outward budding of the membrane [77].

The process of OMVs production is influenced by several factors, from genetic manipulation to environmental factors, such as:

- Iron depletion. Decreased iron availability in *H. pylori* leads to reduced expression of OMVs-associated virulence factors [78].
- Oxidative stress. Exposure to oxidative stress can lead to an increase in the vesicle production, for example, in *P. aeruginosa* the exposure to hydrogen peroxide increases the vesiculation [79].
- Temperature. The temperature to which bacteria are exposed is an important stimulus for the vesiculation, exposure of *Pseudomonas putida* to 55ºC heat shock lead to increased vesiculation [80], however it is also possible to observe an inverse behavior in *Serratia marcescens* in which vesiculation is higher at temperatures ranging 22ºC to 30ºC, while being minimal at 37ºC [81].
- Exposure to chemical products, such as antibiotics or organic solvents. In *P. aeruginosa* the exposure to gentamycin [56] and to polymyxin B [79] increased the amount of vesicles shed. A similar behavior is observed in *Shigella dysenteriae* in which the release of Shiga toxin by OMVs is increased upon exposure to mitomycin C [82].
- Cell-to-cell signaling molecules. Exposure to signaling molecules such as *Pseudomonas* Quinilone Signal (PQS), which is responsible for cell-to-cell signaling and biofilm formation in *P. aeruginosa*, influences the formation of vesicles in both Gram-negative and Gram-positive bacteria [83; 84].

As said before, OMVs production may also be affected by genetic manipulation. Mutations in genes such as RpmM in *N. meningitidis* [85] and DegP in *E. coli* [77] will lead to over-vesiculating mutants.

It is also interesting to notice that the characteristics of OMVs change when exposed to different environmental conditions. OMVs produced during exposure to gentamycin in *P. aeruginosa* were slightly larger than OMVs produced in gentamycin-free conditions [56]. Besides changes in the diameter of the vesicles formed, alterations in LPS of the vesicles may also occur, in *H. pillory* the LPS structure and abundance suffered alterations under iron limiting conditions [86].

The regulation of OMVs production is also related to the biological functions performed by OMVs. These vesicles have several biological roles and are an important mean of survival and interaction with the environment. OMVs have a role in:

- Providing protection during exposure to stresses. During exposure to stressful conditions vesiculation works as an envelope stress response, providing better survival for the cells under stressful conditions [66].
- Facilitate nutrient uptake. OMVs may carry proteins capable of facilitating nutrient acquisition [87], and produce nutrients which become available to surrounding bacteria [88], thus playing an important role in the nutrient uptake by bacteria [89].
- Provide resistance to antibiotics, phages, and toxic substances. OMVs have the ability of providing antibiotic resistance to bacteria, either by acting as a decoy, absorbing antimicrobial or toxic compounds [90; 91], or by packing enzymes capable of mediating antimicrobial activity [92]. Reports suggesting the transfer of DNA by OMVs to other bacteria results in the transference of antibiotic resistance mechanisms. This behavior happens even between different species of bacteria [93].
- Virulence factor delivery. Certain bacteria, such as *P. aeruginosa* and *E. coli*, use OMVs for delivery of virulence factors, such as toxins, to host's cells [67; 56; 94]
- Predatory functions. Certain bacteria are able to produce OMVs capable of killing other bacteria, these vesicles are able to deliver enzymes, such as autolysins, and other compounds, capable of killing other bacteria [97; 98].

The biological functions performed by OMVs allow OMV-producing bacteria to have more success in survival, and in some cases even bacteria belonging to other species benefit from the presence of OMVs [99; 100], thus the capability of producing OMVs is an important mechanism for increased survival and success in the environment.

1.4.3 *In vitro* production and isolation of OMVs

In order to study OMVs, their biogenesis, biological roles, effects in host's and everything that requires their isolation, it becomes necessary to produce and isolate them *in vitro*. Bellow it is described the processes involved in the production and isolation of OMVs in the laboratory.

The process of *in vitro* production and purification of OMVs begins with the culture of the strain from which the vesicles are desired. The culture is performed until late log phase is reached, in which OMVs production is greater [50; 94]. Horstman & Kuehn have described a protocol for the isolation of OMVs from the culture medium, consisting in the culture of the bacterial cells, followed by their

removal by a low-speed centrifugation (10,000 x *g*). The cell-free supernatant was concentrated by ultrafiltration with a 70 kDa tangential flow filtration, followed by another step of low-speed centrifugation (6000 x *g*), to remove any bacterial cell which could be present in the retentate [94]. At this point in the isolation several authors have applied a filtration with 0.22 µm to 0.45 µm in order to remove outer membrane debris and protein aggregates, which cannot be separated from the OMVs by the following process [94; 101; 102]. Finally, in order to obtain the concentrated OMVs, an ultracentrifugation step (39,000 *g* to 150,000 *g*) is applied, which is capable of pelleting the vesicles, which can be resuspended in a small volume of buffer solution, such as HEPES or Tris-HCl [50; 67; 94]. This protocol allows the isolation of OMVs from a bacterial cell culture and is adopted by several authors.

Some alterations may be applied to the isolation process, slightly adapting the protocol to the desired objective. The use of an ultrafiltration is based on the removal of non-OMV-associated proteins from the bacterial supernatant and concentrating OMVs in the retentate, this is achieved by tangentially flowing a bacterial supernatant through a membrane with a molecular weight cut-off between 50 to 100 kDa. The ultrafiltration exhibits highly predictive characteristics, which make this process desirable for the development of products used in the pharmaceutical industry [103].

The alternative to the use of this process is the isolation of the OMVs by precipitation. This method uses ammonium sulfate to aggregate the vesicles, due to disturbance of hydrogen bounds, allowing for an easier isolation by centrifugation [64; 104]. In order to isolate *Bacteroides gingivalis*-derived OMVs it is possible to use a solution saturated with 40% ammonium sulfate to precipitate the vesicles [64; 104]. However it is necessary to carefully control the percentage of ammonium sulfate used, since an increase in concentration will result in the isolation of proteins not associated with OMVs [105]. The use of this method requires the dialysis of the OMVs after their recovery by centrifugation against a suitable buffer, such as HEPES or Tris [64; 104].

1.4.4 Purification and analysis of OMVs

1.4.4.1 Purification processes

The use of isolation processes allows the recovery of OMVs, however these techniques are not able to separate components such as flagella, cable pilus and large protein aggregates from OMVs [50]. The removal of these contaminants is required, particularly for the use of OMVs for vaccine development, in order to obtain a highly pure OMV preparation. Two main purification techniques are used regularly to purify OMV preparations. The first is the use of gradient density centrifugation, and is the most widely used method. The second is the use of Size Exclusion Chromatography (SEC) to separate the components, which is not commonly used, had been used by only a few authors [106; 107].

Purification of OMVs preparations using density gradient centrifugation is performed by loading the OMVs preparation into a density gradient medium, such as iodixanol (Optiprep™), and subjecting the preparation to a centrifugation which allows the separation of the different components according to

their density, which for OMVs is usually lower than the density of pili and flagella [50; 94; 108]. Besides Optiprep™ other density gradient mediums are also available, such as dextran [109] and sucrose [110].

Purification of OMVs preparations is also possible to achieve by the use of SEC. This technique separates particles according to their size as they pass through a SEC medium packed inside a column. This technique is suited for separation of sensitive biomolecules, being possible to be used in a wide range of temperatures (from 37ºC to a cold room), with various co-factors, urea, among other components which may be important to maintain the stability of the biomolecules [111]. There have been reports of successful purifications of OMVs using this technique. In *N.meningitidis* it was possible to purify OMVs by using a Sephacryl S200 chromatography [106], and in *N. meningitidis*, using Sepahrose 6 Fast Flow size exclusion matrix [107].

1.4.4.2 OMVs analysis

Following purification of OMVs it is necessary to quantify and analyze the vesicles, in order to assess both the yield and the efficiency of the purification methods.

The quantification of OMVs yields may be done by protein quantification or lipid quantification. For protein quantification multiple methods are available, from the use of common methods such as Bradford [112] and bincinchoninic acid (BCA) [113], to the use of more complex methods such as the use of densitometry to analyze immunoblotted OMVs preparations [50]. A different approach for the quantification of OMVs yields is the quantification of lipids, which may be performed by using lipophilic dyes which fluoresce upon incorporation into lipid layers [91]. It is also possible to detect components exclusive of LPS, such as 3-deoxy-D-*manno*-octulosonic acid, and quantify them, reflecting the quantity of OMVs present in the sample [81]. Lastly it is possible to use ELISA assays to quantify lipids by using an anti-LPS antiserum [114].

Besides the quantification of OMVs it is also necessary to evaluate the purity and the composition of the sample. The use of Transmission electron microscopy (TEM) allows the visualization of OMVs, thus allowing to analyze the presence of OMVs, their size and shape as well as the possible presence of contaminants. The samples subjected to TEM analysis are stained with uranyl acetate and carefully fixated with glutaraldehyde or formaldehyde [77; 81; 94]. Using this type of microscopy it is even possible to use immune-gold labeling to highlight certain OMVs components [56].

2. Aim of Studies

The present work is integrated in the research project BBi4/2015: “Design and optimization of a scalable manufacturing process for an outer membrane vesicle-based vaccine against *Burkholderia cenocepacia”.* The main goal of this project is to investigate the applicability of *B. cenocepacia*-derived OMV for vaccination purposes.

Burkholderia cepacia complex bacteria are a pathogen associated with opportunistic infections in immunocompromised patients, particularly CF patients. The infections caused by these bacteria are difficult to eradicate due to their intrinsic antibiotic resistance. The difficulties presented in the treatment of the infections caused by these bacteria motivate the development of alternative strategies, such as vaccines. Some research has been performed in the development of vaccines against these pathogens, which were mainly focused in the use of OMP. Recently there has been an increasing interest in the use of OMVs for the development of vaccines against various pathogens, with a vaccine against *N. meningitidis* B having already reached the market (Bexsero™), and a vaccine against *B. pseudomallei* in advanced development stages. These vesicles have characteristics which make them desirable for vaccine development, such as being acellular, multiple antigens are present in their native conformation and they are stable at 4ºC. The use of OMVs may be an attractive strategy for the development of a vaccine against Bcc bacteria, however it requires their isolation and purification. The starting point of this work was the use of *B. cenocepacia* K56-2, a piliated epidemic strain isolated from a CF patient, for the production of *B. cenocepacia*-derived OMVs. After OMVs production several purification steps will be taken and the final OMVs preparations will be characterized in their composition and purity. An additional purification step, using size exclusion chromatography, will also be implemented in the process, with the objective of increasing the purity of OMVs obtained. As OMVs production is strain dependent and varies with the growth conditions, several Bcc strains and growth conditions were evaluated. Moreover, since the main goal of this project is to use OMVs for vaccine development, the purified OMVs obtained in this study, with the purity confirmed by TEM, will be evaluated in cytotoxic assays, both *in vitro* and *in vivo*, namely human bronco epithelial cells (16HBE14o-) and *Galleria mellonella* larvae model, respectively.

In summary, this work aimed at i) the characterization and evaluation of the purity of OMVs isolated from a *B. cenocepacia* culture, ii) the optimization of the production and purification of OMVs, and lastly iii) a preliminary evaluation of cytotoxic potential of purified OMVs.

3. Materials and methods

3.1 Culture conditions and bacterial strains

In this work 10 different bacterial strains were used, detailed information of these strains can be seen in Table 3.1. The long term storage of the isolates was performed by freezing the samples in glycerol at -80ºC. When necessary strains were defrost to Luria-Bertani (LB) (Nzytech, Lisbon, Portugal) plates and grown at 37ºC for 24 hours. Culture plates were used to pre-inoculate 100 mL of LB medium and grown overnight at 37ºC and 250 rpm orbital agitation.

3.2 OMVs production

For the production of OMVs *B. cenocepacia* overnight cultures were used to inoculate two Erlenmeyer flasks containing 1 L of LB medium, with an initial OD_{640nm} of 0.1. The cultures were grown at 37ºC and 250 rpm orbital agitation until an OD_{640nm} of 1.5 was reached, which lasted approximately 5 to 6 hours, corresponding to the final stage of exponential growth.

3.3 OMVs purification

Upon finishing the OMVs production, the samples were subjected to a downstream processing, in order to remove contaminants and to concentrate the vesicles. The protocol used for OMVs isolation was based on several experimental procedures previously described [115; 116] and will be presented in the next sections.

3.3.1 Bacterial cell removal

The purification of the OMV samples begins with the removal of bacterial cells from the culture medium, which was achieved by centrifugation at 10,000 x *g* during 10 minutes, at 4ºC (Beckman J2-MC). The supernatant was collected and the pellet was discarded. In order to completely remove the bacterial cells from the supernatant, it was filtered through a 0.45 µm cellulose membrane (Milipore, Bedford, MA, USA). The cell-free supernatant was stored at 4ºC until use in the next purification step.

Table 3.1- Characteristics of the Bcc isolates used in this work.

Strain name	Other name	Source, location	Relevant information	Source
Burkholderia cenocepacia				
K56-2	LMG 18863	CF patient, Canada	ET-12 lineage BCESM$^+$; *cblA*$^+$; *recA-A*	J. J. LiPuma
J2315	LMG 16656	CF patient, UK	ET-12 lineage BCESM$^+$; *cblA*$^+$; *recA-A*	G. Doring
AU1054	LMG 24506	CF patient, USA	*recA-B* PHDC lineage	BCCM™/LMG
MCO-3	LMG 24308	Maize rhizosphere, USA	*recA-B*	BCCM™/LMG
HI2424	LMG 24507	Soil, USA	*recA-B* PHDC lineage	BCCM™/LMG
Burkholderia multivorans				
ATCC17616		Soil, USA		ATCC
D2095		CF patient, Canada		D. P. Speert
HI2229	LMG 17588	CF patient, USA	BCESM$^-$; *cblA*$^-$	J. J. LiPuma
Burkholderia cepacia				
PC783	LMG 1222	Onion, USA		J. J. LiPuma
Burkholderia dolosa				
AU0654	LMG 18943	CF patient, USA		J. J. LiPuma

BCESM: *Burkholderia cenocepacia* epidemic strain marker – *cblA:* cable pilus gene – *recA*: recombinase A gene – PHDC: Philadelphia-District of Columbia clone – ET: Electrophoretic type

3.3.2 Ultrafiltration and diafiltration

The cell-free supernatants were concentrated by ultrafiltration, which allowed the concentration of 2 L to approximately 70 mL. Ultrafiltration was performed using 100,000 Da nominal molecular weight cut-off (NMWC) hollow fiber cartridge with a membrane area of 650 cm^2 (GE Healtcare, Westborough, MA, USA). The working pressure was 10 psi, achieved by setting the pump's rpm to a fixed value (200 rpm) and by adjusting the valve at the concentrate outlet. Pressure was adjusted during the process as necessary in order to maintain a constant pressure of 10 psi. The permeate flow rate was measured every 500 mL, to evaluate the fouling of the membrane. After processing the 2 L of sample and achieved 200 mL of retentate a diafiltration was started by adding 4 L of phosphate buffered saline (PBS) (pH 7.4) at 4ºC, to replace the extracellular medium . The diafiltration was also performed at 10 psi and the process was finished when a minimal volume of 70 mL was achieved. At the end of

this process the retentate would contain molecules with more than 100 kDa, such as OMVs or other large proteins.

After the process of ultrafiltration and diafiltration is necessary to regenerate the membrane, in order to remove the fouling accumulated during the process. The regeneration of the membrane was performed at a pressure of 5 psi, as indicated by the manufacturer, by alternating the flow-through of 10% (v/v) isopropanol (Fisher Scientific, UK) and 0.1 M of sodium hydroxide solution (VWR Chemical, Belgium), in the filtration system. Each solution was left to run through the membrane for one hour and deionised water was used to remove all the residues between solutions. Several regeneration cycles were performed until the permeate flow rate reached a value similar to the beginning of the ultrafiltration, measured with deionised water. The storage of the hollow fiber cartridge was performed in a solution of 10% (v/v) isopropanol.

3.3.3 Ultracentrifugation

For further concentration of the sample after the ultrafiltration/diafiltration, an ultracentrifugation was performed. Ultracentrifugation was performed at 150,000 x *g* during 3 hours, at 4ºC (Beckman XL-90, Brea, CA, USA). Supernatant was discarded and the vesicles pellet was resuspended in 1.5 mL of PBS (pH 7.4). OMVs solution was stored at 4ºC. When sterile conditions were needed OMVs solutions were filtered with a 0.2 µm polyethersulfone membrane (Whatman, GE Healthcare, Life Sciences, UK).

3.3.4 Size Exclusion Chromatography

To test another purification step, OMVs solutions obtained from the ultracentrifugation were subjected to a size exclusion chromatography, using a Tricorn™ 5/100 or Tricorn™ 10/600 column packed with 15 mL or 35 mL of Sephacryl S-1000 Superfine (GE Healthcare Life Sciences), respectively.

The matrix of this gel filtration medium is achieved by covalently cross-linking allyl dextran with N,N'-methylenebisacrilamide with a particle size between 40 and 105 µm, allowing the purification of sperichal particles up to 400 nm.

All size exclusion chromatography runs were performed using an Äkta™ Purifier 10 system (GE Healthcare, Uppsala, Sweden). Data collection and analysis was performed using UNICORN control software (GE Healthcare, Uppsala, Sweden), continuously monitoring parameters such as UV absorbance at 280 nm, pressure, flow rate and temperature.

Before injection of the sample, the column was equilibrated with 2 column volumes of PBS (pH 7.4), using a flow rate of 1 mL/min. Upon the equilibration of the column, 1.5 mL of OMVs solution was loaded using a 1 mL sample loop with a flow rate of 0.5 mL/min. After the injection of the sample a isocratic elution was performed with 2 column volumes of PBS, the flow-through and the elute were collected continuously as 0.5 mL fractions in a FRAC 950 Fraction collector (GE Healthcare, Uppsala, Sweden).

The buffers used in the chromatographic steps were subjected to a filtration through a cellulose membrane with a pore size of 0.45 µm (Milipore, Bedford, MA, USA).

3.4 Evaluation of OMVs production under different growth conditions and strains

Different strains were pre-inoculated overnight in 100 mL of LB broth (Nzytech, Lisbon, Portugal), at 37ºC and 250 rpm orbital agitation. Overnight cultures were used to inoculate 150 mL of LB medium with an initial OD_{640nm} of 0.1. The cultures were grown at 37ºC and 250 rpm orbital agitation until an OD_{640nm} of 1.5 was reached.

Following the culture, bacterial cells were removed by centrifugation at 10,000 x *g* during 10 minutes, at 4ºC (Beckman J2-MC, Bread, CA, USA). The supernatant was filtered through a 0.45µm cellulose membrane (Milipore, Bedford, MA, USA). The cell-free supernatant was collected and concentrated using a centrifugal filter Amicon® (Merck Millipore, Cork, Ireland) with 100,000 Da NMWL, until a final volume of 1.5 mL was reached.

The analysis of OMV production along the culture growth was performed for the strains *B. cenocepacia* K56-2 and *B. multivorans* HI2229. The protocol used for production and purification of OMVs was similar to the one previously described in this section, differing only in the culture volume (500 mL) and in time of growth, 4, 10 and 16 hours of incubation, as well as at an OD_{640nm} of 1.5.

The analysis of the effect of different growth conditions in OMVs production was performed by culturing and purifying the samples as described previously in this section, only altering the volume of culture to 500 mL. Two different mediums were used, regular LB broth (Nzytech, Lisbon, Portugal) and LB medium with 300 mM of NaCl (VWR Chemicals, Belgium). Two different culture conditions were also tested, microaerophilic conditions and normoxia. The culture grown in microaerophilic conditions required different incubation conditions, the initial OD_{640nm} used was 0.2, the inoculated medium was distributed in petri dishes and placed inside an anaerobic box (Mitsubishi Gas Chemical Company, Japan) with a GENbox microaerophilic generator (BioMérieux, Marcy l'Etoille, France). The box was incubated at 60 rpm orbital agitation and 37ºC, during 17 hours, reaching an OD_{640nm} of 1.5. Following the cultures the processing of the culture medium was performed as previously described in this section using a centrifugal filter Amicon® (Merck Millipore, Cork, Ireland).

3.5 OMVs analysis

3.5.1 Proteomic analysis

The 2-Dimensional Gel Electrophoresis (2-DE) was performed by Dr. Pedro M. Santos at CBMA-Centre of Molecular and Environmental Biology, Department of Biology, University of Minho, Braga, Portugal. Briefly, three independent *B. cenocepacia* K56-2 OMVs samples were mixed and analyzed. Labeling buffer (20 mM Tris, 7 M urea, 2 M thiourea, 1% (w/v) dodecylmaltoside) was added to

samples to solubilize the membrane proteins. Isoelectric focusing was performed using the immobilized pH gradient technique (IPG) with non-linear 24 cm IPG strips (pH 3 to 10) with the reducing buffer [7 M urea (Sigma Aldrich), 2 M thiourea (Sigma Aldrich), 1% (w/v) dodecylmaltoside (Sigma Aldrich), 0.002% (w/v) bromophenol blue (Sigma Aldrich), 1.2% (v/v) DeStreak (GE Healthcare, Uppsala, Sweden) and 0.5% (v/v) pharmalytes (GE Healthcare, Uppsala, Sweden)]. The isoelectric focusing (IEF) step was carried out for 3 h focusing with a total voltage of 7,000 V applied and the IPG strips equilibrated in reducing buffer for 20 min under agitation in 30% (v/v) glycerol (Sigma Aldrich), 2% sodium dodecyl sulphate (SDS), 6 M urea, 50 mM Tris and 2% dithiotreitol (DTT) (Sigma Aldrich). The IPG strips where then alkylated in the same buffer containing 2.5% (v/v) iodoacetamide (Sigma Aldrich). The IPG strips were placed on 12% dodecyl sulfate polyacrylamide gel (SDS-PAGE) using the EttanDaLTsix instrument (GE Healthcare, Uppsala, Sweden) and the separation was carried out at 110 V. Analytical gels were scanned on a Typhoon Trio laser scanner (GE Healthcare, Uppsala, Sweden) and preparative gels were stained with silver nitrate.

Protein spots were excised manually from polyacrylamide gels and send for identification by mass spectrometry (MS) in the Proteomic and Analytical Biochemistry Laboratory, Department of Biology, University of York, York, UK. Briefly, proteins in gel spots were digested with trypsin, and tryptic peptides analyzed by matrix-assisted laser desorption ionization (MALDI)-MS and MS/MS using a Bruker ultraflex III (Bruker-Daltonic, USA) tandem time of flight (TOF/TOF) mode. A maximum of 10 MS/MS spectra were acquired for each sample. Mass spectra were matched to NCBI non-redundant protein database, using MASCOT software (Matrix Science, London, UK) to match proteins. The classification into functional categories was performed using the Burkholderia genome database (http://www.burkholderia.com), TIGR database (http://www.tigr.org/), the Role Category Lists, KEGG database (http://www.genome.jp/kegg/) and NCBI (http://www.ncbi.nlm.nih.gov/). Subcellular prediction was performed using the bioinformatic tool PSORTb version 2.0.4 [117]

3.5.2 Total protein quantification – BCA assay

OMVs purified according to the previously described procedures were subjected to total protein quantification, performed by a bicinchoninic acid (BCA) assay, using a Pierce BCA protein kit (Pierce, Rockford, IL, USA).

Standard solutions of bovine albumin serum (BSA) ranging from 25 to 450 µg/mL were used to establish a calibration curve.

Assays were performed in 96 wells microplates using 200 µL of BCA kit reagent and 10 µL of the sample. Plates were incubated in the dark for 15 min and absorbance at 562 nm was read in a Spectrostar^{nano} (BMG Labtech, Germany) microplate reader.

3.5.3 Protein gel electrophoresis

During the ultrafiltration/diafiltration process, samples of the different flows were collected to evaluate the protein content. Samples of 5 mL from the retentate and permeate were collected in the beginning and end of the process. Following collection, samples were concentrated 100x, for a final

volume of 50 µL, using centrifugal filter Amicon® (Merck Millipore, Cork, Ireland) with 3,000 Da NMWL. The centrifugation was performed at 14,000 x *g* during 10 minutes at 4ºC (Sigma 2K15, Germany).

Concentrated samples were analyzed by SDS-PAGE, allowing the evaluation of the molecular size of proteins present in the sample. For each 30 µL of laemli sample buffer (Bio-Rad, USA) containing 0.1 M DTT was added to 20 µL of each sample, and boiled for 10 min. at 100ºC.

Samples were loaded into a 12% SDS-PAGE and run at 90 mV using the running buffer containing [25 mM Tris-HCl (Sigma Aldrich), 192 mM glycine (Sigma Aldrich) and 0.1% (w/v) SDS (Sigma Aldrich) at a pH of 8.3]. A Precision Plus Protein™ Dual Color Standard molecular marker (Bio-Rad, USA) was used as a molecular weight marker.

The staining of the gel was performed by silver staining.

The process started with the fixation by incubating the gel with a 30% (v/v) ethanol and 10% (v/v) acetic acid during 2 hours. Then it was washed with 30% (v/v) ethanol and deionized water. A sensibilization step was performed by incubating the gel during 15 min with a solution of 0.02% (v/v) sodium thiosulfate (Sigma Aldrich), followed by three washing steps with deionized water. The staining was performed by incubating the gel during 30 min with a 0.15% (w/v) silver nitrate (Sigma Aldrich). Upon staining, the gel was incubated with a development solution to reveal the bands corresponding to the protein fractions present in the samples. The development solution was freshly prepared with 3% (w/v) sodium carbonate (Sigma Aldrich) and 0.05% (v/v) formaldehyde (Sigma Aldrich). As soon as the bands were defined a solution of 5% (v/v) acetic acid was poured in the gel and incubated during 15 min to stop the reaction.

3.5.4 Cytotoxic assays

3.5.4.1 MTT Assays

The MTT [3-(4,5 dimethylthiazol-2-yl-2,5 tetrazolium bromide)] assays were used to evaluate the occurrence of cytotoxic effects on 16HBE14o- human bronchial epithelial cells [118] upon exposure to purified OMVs.

Cells were mantained in fibronectin/vitrogen coated flasks in Minimum Essential Medium with Earle's salt (MEM) (Gibco, Thermo Fisher Scientific, Rockford, IL, USA) supplemented with 10% fetal bovine serum (FBS) (Lonza, Switzerland), 0.292 g/L L-glutamine (Sigma Aldrich) and Penicilin/Streptomycin 100 U/mL (Gibco, Thermo Fisher Scientific, Rockford, IL, USA) in a humidified atmosphere at 37ºC with 5% CO_2. One day prior to treatment with OMVs cells were seeded in 96-well plates (Orange Scientific, Belgium) at a density of $7.5x10^4$ cells per well. Different quantities, 0.5 µg, 1 µg and 2 µg of a *B. cenocepacia* AU1054-derived OMVs were added to 100 µL of MEM medium and incubated during 24 h, at 37ºC, with 5% CO_2. After that time 20 µL of 5 mg/mL MTT were added to each well and the plate incubated at 37ºC during 3 hours. Following incubation, the medium containing MTT was removed and 150 µL of 40 mM HCl in isopropanol was added in order to stop the reaction. The plate was incubated during 15 min, with soft agitation at room temperature and the crystals ressuspended. Plates were analyzed at 590 nm in a SpectroStar^{nano} (BMG Labtech, Germany)

microplate reader. Untreated cells were used as control, to allow for the determination of the relative cell viability of the treated cells.

3.5.4.2 Galleria mellonella killing assays

Galleria mellonella larvae model was used to evaluate *in vivo* cytotoxic effects of *B. cenocepacia* AU1054-derived OMVs. *G. mellonella* larvae were reared in an insectarium on a beeswax and pollen grain diet, at 25ºC, in darkness. Last instar larvae weighing 250 ± 25 mg were used for these assays.

A micrometer was adapted to control the volume of a microsyringe and inject 3.5 µL (0.2 µg) or 10 µL (1 µg) of purified OMVs in each caterpillar via the hindmost left proleg, previously sanitized with 70% (v/v) ethanol. Following injection, larvae were placed in Petri dished and incubated in the dark, at 37ºC.

For each condition, 10 larvae were used to follow daily larval survival during a 96 hours period. Caterpillars were considered dead when they displayed no movement in response to touch. Control larvae were injected with PBS (pH 7.4).

3.5.5 Transmission Electron Microscopy

To assess OMVs purity, samples were analyzed by Transmission Electron Microscopy. Ten microliters of purified OMVs were negatively stained with freshly prepared 2% (v/v) uranyl acetate and applied to 200-mesh carbon-coated cooper grids (Electron Microscopy Sciences, Hatfield, PA, USA). The samples were then visualized with a JEOL 1200-EX (Tokyo, Japan) transmission electron microscope. TEM analysis was performed in collaboration with Dr. António Pedro Alves de Matos, at Centro de Investigação Interdisciplinar Egas Moniz- (CiiEM), Egas Moniz- Cooperativa de Ensino Superior, Caparica, Portugal.

4. Results

4.1 *In vitro* production of OMVs using *Burkholderia cenocepacia* K56-2 strain

It is known that OMVs are abundantly shed by *B. cenocepacia* strains grown in culture medium [116]. In this work the production of OMVs from *B. cenocepacia* K56-2 strain was assessed during *in vitro* culture. Bacteria were cultured in LB medium, under aerobic conditions until an OD_{640nm} of 1.5 was reached. OMVs were isolated from culture supernatants, which were subjected to an ultrafiltration and ultracentrifugation to purify and concentrate the OMVs, respectively (Fig. 4.1). To quickly evaluate the amount of OMVs obtained after the purification steps, a quantification of the total protein was performed and used as an indicator of the OMVs produced. Starting with 2 L of culture it could be obtained 0.1 ± 0.03 mg/L of initial culture, of total protein in the purified vesicles solution.

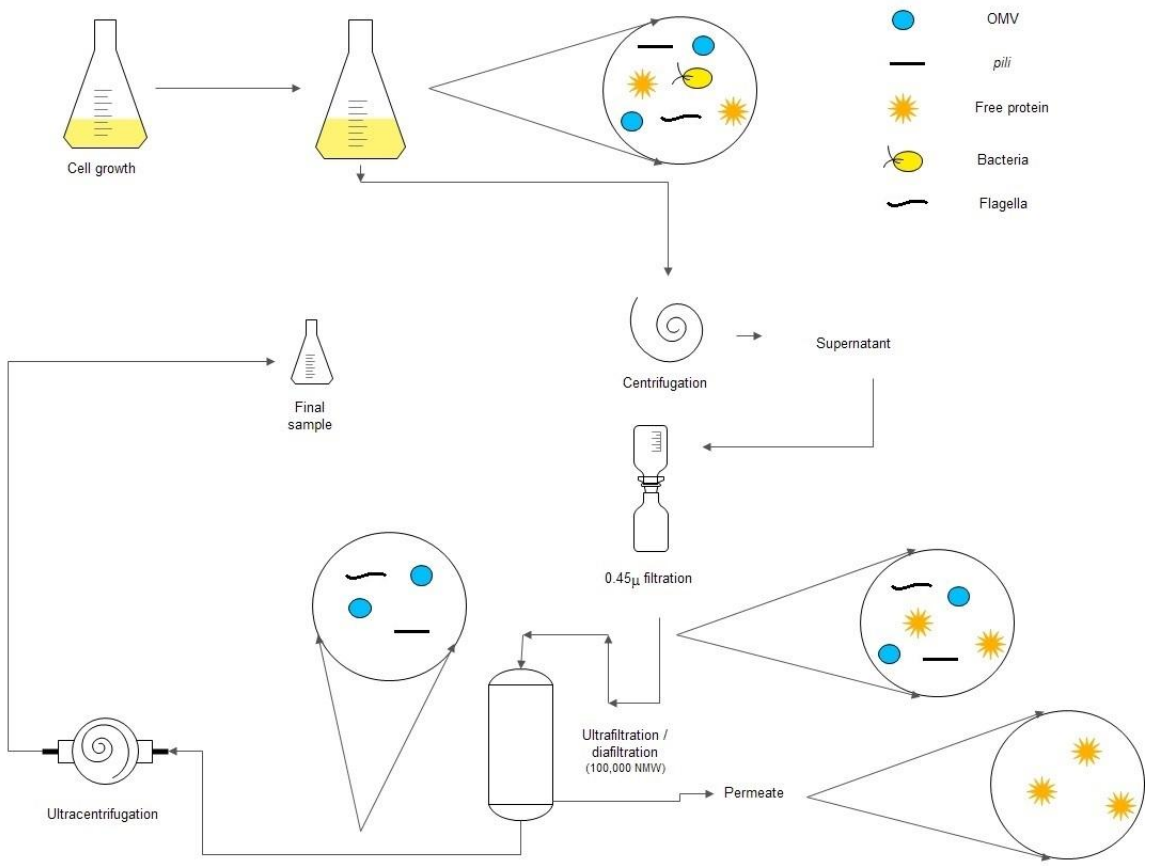

Fig. 4.1- Diagram of OMV production and purification process.

These vesicles were examined by TEM, revealing the presence of typical spherical bilayer structures with diameters ranging from 10 to 300 nm. The small-sized OMVs with diameters of approximately 50 nm were commonly observed, whereas relatively large-sized vesicles with diameters superior to 150 nm were less common.

The presence of contaminants namely flagella and cable pili was also observed (Fig. 4.2). The presence of such contaminants in the *B. cenocepacia* K56-2-derived OMVs renders impracticable the application for vaccine development, thus requiring a more rigorous purification procedure. In order to achieve this objective, different approaches may be tested, from the substitution of the strain used for OMV production (by a strain non producer of cable pili), or by enhancing the purification process, applying a process capable of removing the contaminants more efficiently.

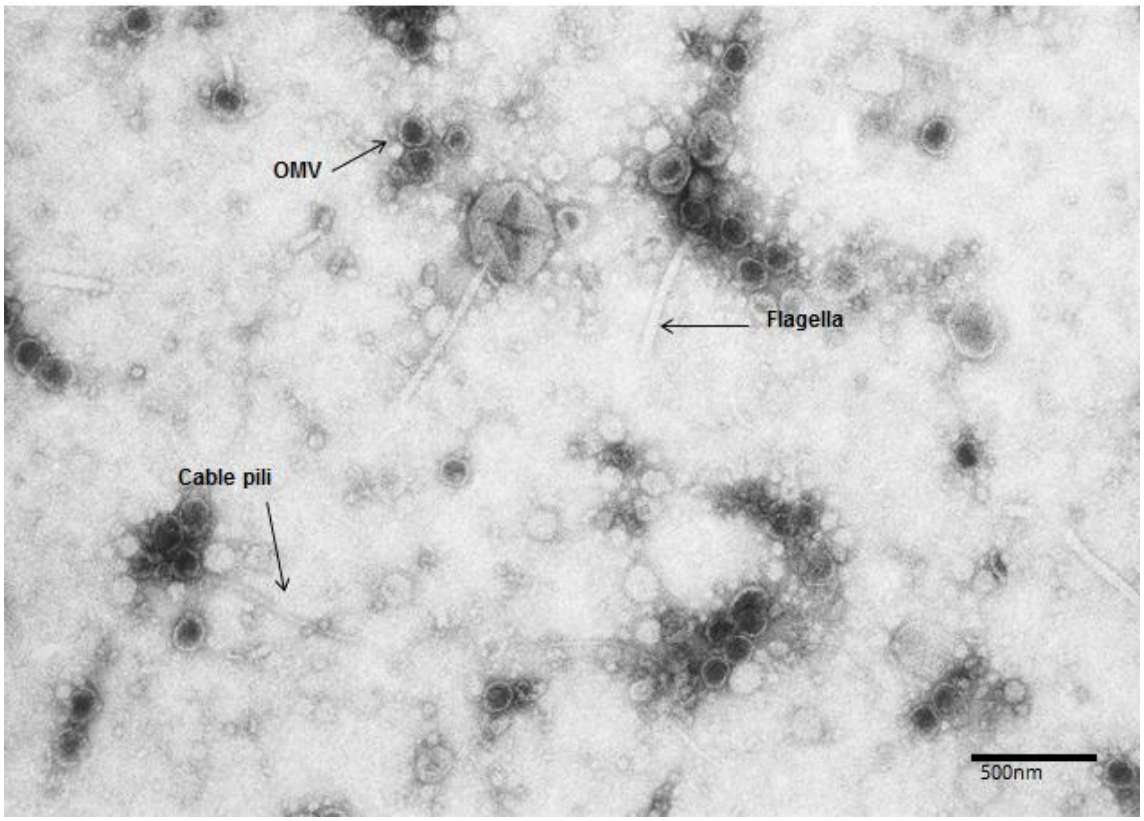

Fig. 4.2 - TEM analysis of OMV samples obtained from *B. cenocepacia* K56-2 *in vitro* growth.

4.2 Proteomic analysis of *B. cenocepacia* K56-2 derived OMVs

In order to identify the proteins associated with the *B. cenocepacia* K56-2-derived OMVs, three independent samples of purified OMVs were produced as described in (Fig. 4.1) and subjected to a proteomic analysis. The major protein components of OMVs samples were separated by 2-DE and the visible protein spots analyzed by MALDI-TOF/MS, in order to identify the proteins associated with OMVs.

A total of 73 spots where selected in the gel for further protein identification (Fig. 4.3). From these spots, 33 distinct proteins were identified (Table 4.2). Several proteins produced more than one spot due to the isoelectric point or mass variation. The identified proteins were grouped according to the functional category, encompassing 8 functional categories (Table 4.2). The distribution of proteins between the groups was not proportional, the most abundant groups were the cell envelope

biogenesis and outer membrane, the energy production and conversion group and the amino acid transport and metabolism. Proteins with unknown function also represented a large group.

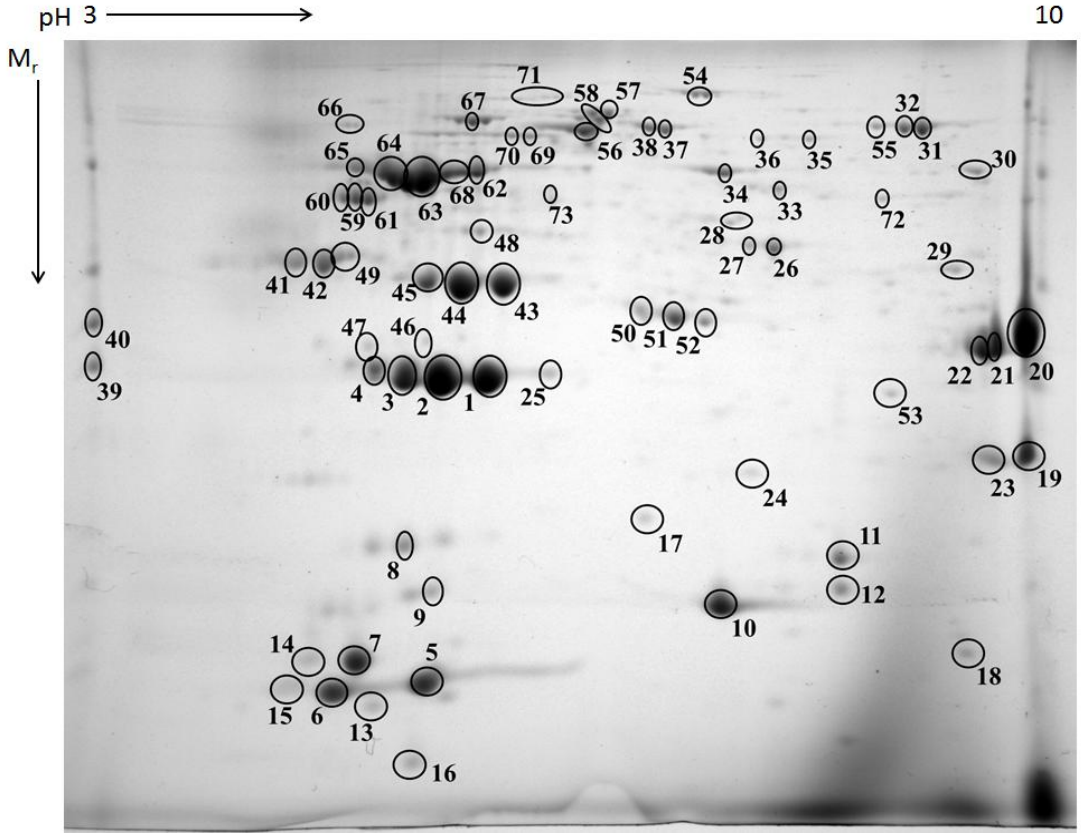

Fig. 4.3- Proteome reference 2-DE gel of *B cenocepacia* K56-2-derived OMVs. On the left is represented the scale of the molecular weight (higher on top to lower on bottom), and on the top is represented the pH scale (from 3 to 10). The circles and numbers indicate excised protein spots which were analyzed by MALDI-TOF.

As expected, membrane-associated proteins are well represented in OMVs. A large number of cytoplasmic proteins are also present which are common in OMVs composition since vesicles are derived from multiple bacterial compartments not only from the outer membrane. The presence of extracellular proteins such as cable pilus and flagella (Table 4.2) corroborates the TEM results (Fig. 4.2), where these contaminants were also observed.

Table 4.1- Proteins of *B. cenocepacia* K56-2-derived OMVs identified by 2-DE and MALDI-TOF MS/MS analysis.

Spot	Protein name	Predicted subcellular localisation	Acession number	MW(kDa) / pI
	Cell envelope biogenesis, outer membrane			
11, 12	OmpW	OM	gi|107029064	22.5 / 8.67
19	OmpA	OM	gi|107022117	37.5 / 9.44
20, 21, 22, 40	porin	OM	gi|206563776	24.2 / 9.45
52	porin	OM	gi|107022430	40.3 / 7.1
31, 32, 55	outer membrane protein assembly complex, YaeT	OM	gi|170733367	85.0 / 8.8
	Energy production and conversion			
26, 27	type II citrate synthase, gltA	CP	gi|53716142	48.9 / 6.32
34	succinate dehydrogenase flavoprotein subunit, sdhA	CPM	gi|78062997	64.9/ 6.3
54	2-oxoglutarate dehydrogenase E1 component, suca	CP	gi|107022581	107.0 / 6.12
57	NADP-dependent malic enzyme	CP	gi|115350586	83.4 / 5.85
58	malate dehydrogenase	CP	gi|107024332	81.6 / 5.66
70	dihydrolipoamide dehydrogenase	CP	gi|107027254	62.4 / 5.36
	Amino acid transport and metabolism			
37, 38	Orn/Lys/Arg decarboxylase, speF	CP	gi|206562965	81.8 / 6.03
56	Orn/Lys/Arg decarboxylase, major domain protein adiA	CP	gi|206562966	82.4 / 5.62
24	imidazoleglycerol-phosphate dehydratase, hisB	CP	gi|158343889	21.4 / 6.29
68	L-glutamine synthetase, glnA	CP	gi|78066927	52.5 / 5.25
50, 51	ketol-acid reductoisomerase, ilvC	CP	gi|78067059	36.4 / 6.02
28	Peptidase C26	CP	gi|78065609	28.0 / 6.31
	Posttranslational modification, protein turnover, chaperones			
63, 64	chaperonin groEL	CP	gi|206561490	57.0 / 5.13
8	hydroperoxide reductase AhpC/TSA family protein, peroxiredoxin	CP	gi|53719707	20.5 / 5.05

Table 4.1- Proteins of *B. cenocepacia* K56-2-derived OMVs identified by 2-DE and MALDI-TOF MS/MS analysis (Continuation).

Spot	Protein name	Predicted subcellular localisation	Acession number	MW(kDa) / pI
	Translation, ribosomal structure and biogenesis			
53	30S ribosomal protein S2 BCAL2091 rpsB	CP	gi|78066798	27.1 / 9.68
48	Elongation factor Tu	CP	gi|416939	43.1 / 5.4
67	pnp polynucleotide phosphorylase/polyadenylase	CP	gi|78067050	77.2 / 5.34
71	phenylalanyl-tRNA synthetase subunit beta, pheT	CP	gi|107022553	89.1 / 5.39
	Carbohydrate transport and metabolism			
49	phosphopyruvate hydratase	CP	gi|53719880	45.9 / 4.76
	Cell Motility			
41, 42	Flagellin	Ext	gi|4210944	38.6 / 4.84
7, 14	giant cable pilus	Ext	gi|206564599	17.0 / 5.36
5, 6, 15	fimbrial protein	Ext	gi|107022699	17.4 / 6.18
	Unknown			
10	hypothetical protein	U	gi|206562922	16.3 / 6.12
13	hypothetical protein	U	gi|206562930	15.6 / 5.05
59, 60, 61	hypothetical protein	CP	gi|206562919	58.6 / 5.6
16	hypothetical protein KS10_gp30	U	gi|198449292	11.5 / 5.17
17	Phasin	U	gi|78066909	19.8 / 5.96
1, 2, 4, 25, 39	putative exported phage protein	U	gi|206562923	37.5 / 5.73

OM – Outer membrane; **CP-** cytoplasmic; **Ext-** Extracellular; **CPM-** Cytoplasmic membrane

4.3 Optimization of growth parameters, conditions and strains used for OMVs production using Bcc strains

4.3.1 Evaluation of OMVs yields using different Bcc strains

It is known that different Bcc strains yield different amounts of OMVs [116]. In order to evaluate the OMVs production by different *B. cenocepacia* strains, 5 strains were tested to verify the existence of a strain capable of producing larger amounts of OMVs than K56-2 strain. This evaluation was also performed with strains from other Bcc species, thus incorporating strains from the four most relevant Bcc species in CF patients infections, more precisely *B. cenocepacia, B. multivorans, B. cepacia* and *B. dolosa* [10; 119].

The OMV production was evaluated by culturing the strains in 150 mL of LB medium until an OD_{640nm} of 1.5 was reached. Following the culture, bacteria was removed from the culture medium, by centrifugation, and the cell-free supernatant was then subjected to a simplified purification and concentration step using centrifugal filters Amicons®. Following the purification, the total protein in the OMVs sample was quantified and used as an indicator of the vesicles produced.

From the quantification of total protein, it was possible to calculate the yield of OMVs produced by liter of initial culture medium (mg of OMVs/ L), for the tested strains (Fig. 4.4). The strain K56-2 exhibited one of the best yields and any strain was able to achieve a significantly better yield. For *B. multivorans* strains the ATCC17616 strain lead to the higher yield, and similar to *B. cenocepacia* K56-2, however it represents an environmental isolate.

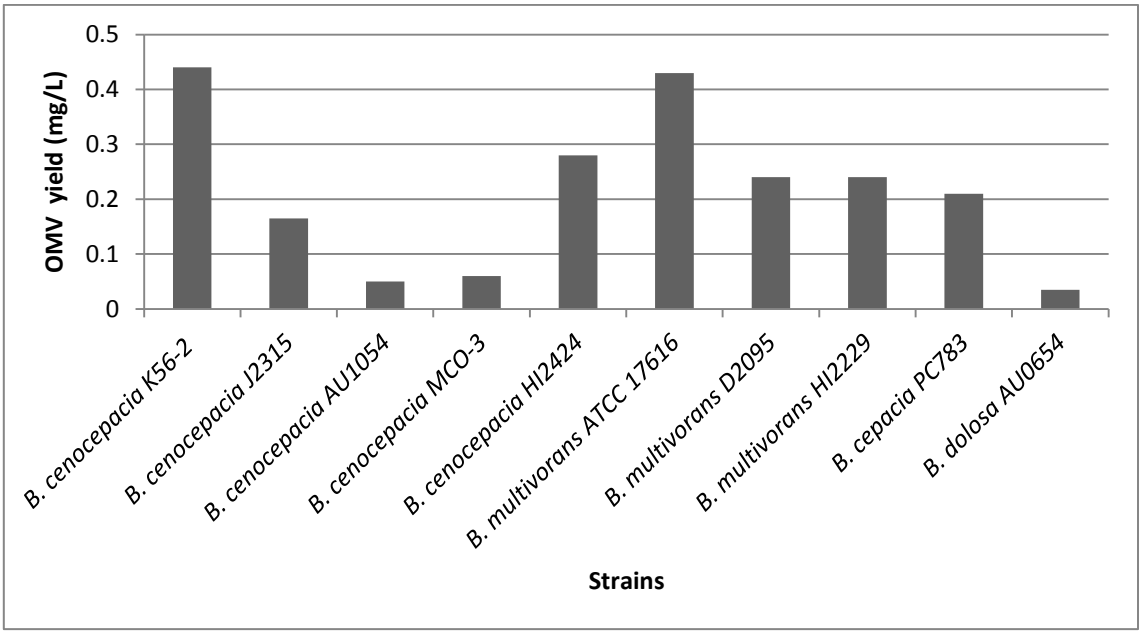

Fig. 4.4- Graphic representation of the total protein amount (mg/L of initial culture) produced by the different Bcc strains used in the screening.

4.3.2 Evaluation of OMV yields along the growth curve

For further optimization of the OMVs yields, the production of OMVs was evaluated in different points of the growth curve. This evaluation was performed using *B. cenocepacia* K56-2 and *B. multivorans* HI2229, in order to have the best yields possible and clinical isolates also represented.

The protocol used for this study was slightly different from the protocol used for the evaluation of OMVs yields by different strains. In this study the bacteria were cultured in 500 mL of LB medium and the culture was stopped at different points of the growth, namely, at 4, 10 and 16 hours, as well as OD_{640nm} 1.5. Upon finishing the culture, the bacteria were removed from culture medium, by centrifugation, and the cell-free supernatant was subjected to a simplified purification and concentration using centrifugal filters Amicons®. Following the concentration and purification, total protein obtained was quantified and used as an indicator of OMVs present.

OMVs yield from *B. cenocepacia* K56-2 was higher for longer incubation periods. After the point representing OD_{640nm} 1.5 (7 hours), the OMVs yield increases, while between the point representing 4 hours of growth and OD_{640nm} 1.5, the increase in OMVs yield was lower (Fig. 4.5).

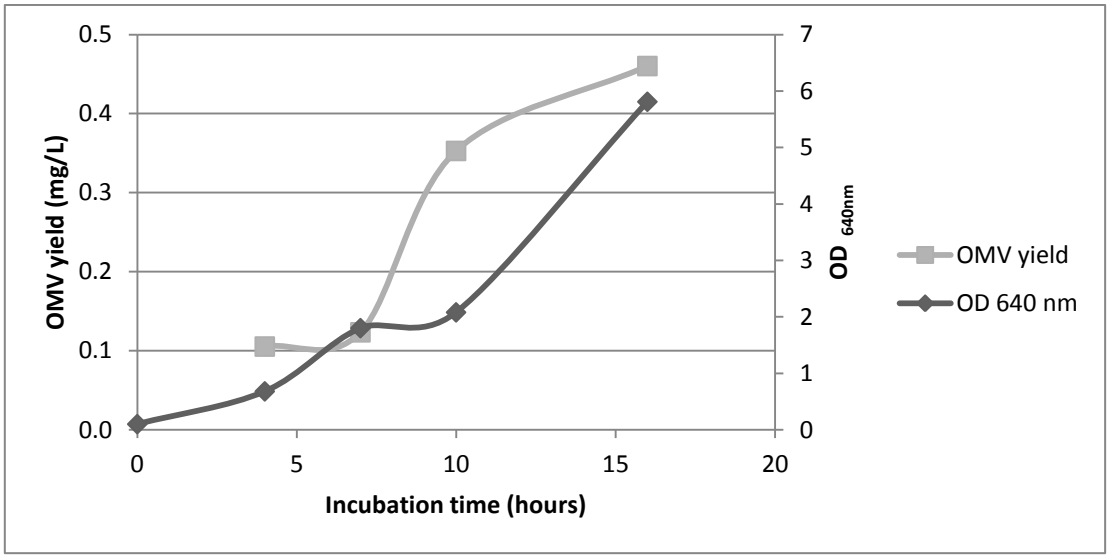

Fig. 4.5- Representations of the OMVs yield (mg/L of initial culture) and the optical density at 640 nm of *B. cenocepacia* K56-2 at different points in the growth curve.

A similar behavior was observed for *B. multivorans* HI2229, achieving higher yields for longer incubation periods (Fig. 4.6).

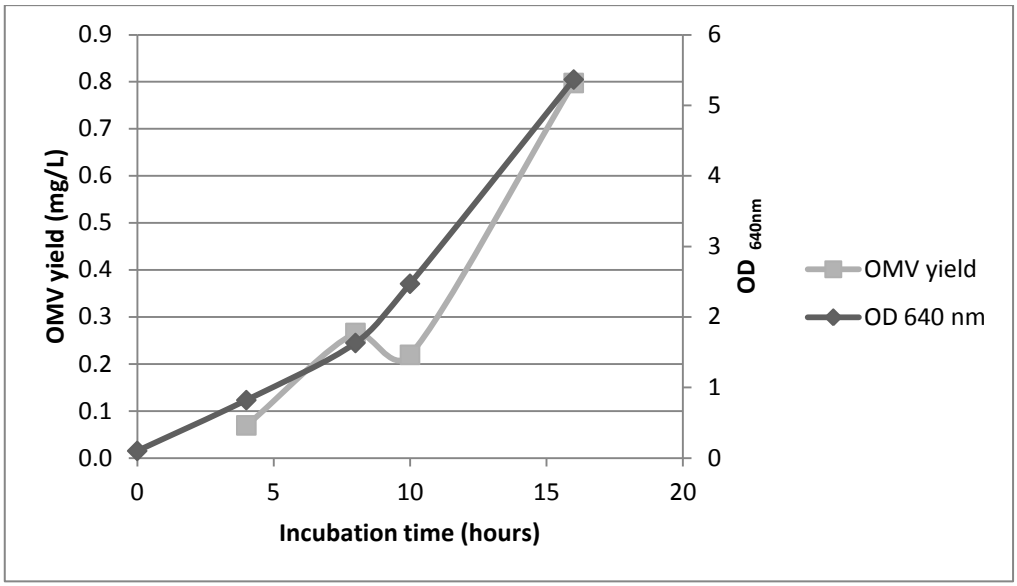

Fig. 4.6 Representations of the OMVs yield (mg/L of initial culture) and the optical density at 640nm of *B. multivorans* HI2229 at different points in the growth curve.

4.3.3 Evaluation of the effect of different growth conditions in the OMVs yields by B. cenocepacia K56-2

It is known that environmental conditions and stresses affect OMV production [77,79]. In order to evaluate the effect of different growth conditions, *B. cenocepacia* K56-2 was cultured under growth conditions based on stresses to which bacteria are subjected when colonizing CF patients lungs, such as osmotic stress, represented by medium with 300 mM of NaCl, and reduced oxygen availability, represented by microaerophilic conditions [120; 121].

This evaluation was performed by inoculating 500 mL of medium with *B. cenocepacia* K56-2 and incubating the bacteria until an OD_{640nm} of 1.5 was achieved. Once OD_{640nm} reached 1.5, the bacterial culture was subjected to the same processing as the cultures used for the evaluation of OMVs yield using different strains.

It can be observed in Table 4.2 that the microaerophilic conditions present the highest OMV yield. The culture subjected to osmotic stress (LB with 300 mM NaCl) resulted in lower yields than the culture using regular LB medium.

Table 4.2- OMV yields (mg/L of initial culture) obtained following culture of *B. cenocepacia* K56-2, under different growth conditions.

Growth conditions			OMVs yield (mg/L)
Broth	**Stirring (rpm)**	**Oxygen**	
LB	250	aerobic	0.9
300 mM NaCl LB	250	aerobic	0.7
LB	60	microaerophilic	1.5

4.4 Monitoring of OMVs during downstream processing of *B. cenocepacia* K56-2-derived OMVs

In order to obtain purified *B. cenocepacia* K56-2-derived OMVs it is necessary to subject the culture medium to a series of purification processes, to remove the bacterial cells and impurities as well as to concentrate the vesicles (Fig. 4.1). During the purification process changes in the composition, and concentration of OMVs, in the cell-free supernatant, may occur, and for that purpose samples were taken from the two streams of ultrafiltration (permeate and retentate), at the beginning and at the end of the ultrafiltration. The recovered samples were concentrated to similar volumes, total protein was quantified and the samples were subjected to SDS-PAGE.

During the ultrafiltration step a substantial reduction in the total protein amount occurs. It is also possible to see that high amount of proteins are wasted in the permeate (Fig. 4.7). These results suggest that small soluble extracellular proteins have been removed from the cell-free supernatant, when subjected to the ultrafiltration, leaving larger proteins, such as cable pilus, flagella and OMVs, in the concentrated OMV sample.

The last step in OMVs purification is an ultracentrifugation to concentrate the OMVs sample. In fact, after this process the sample exhibited a higher protein concentration, which was expected (Fig. 4.7).

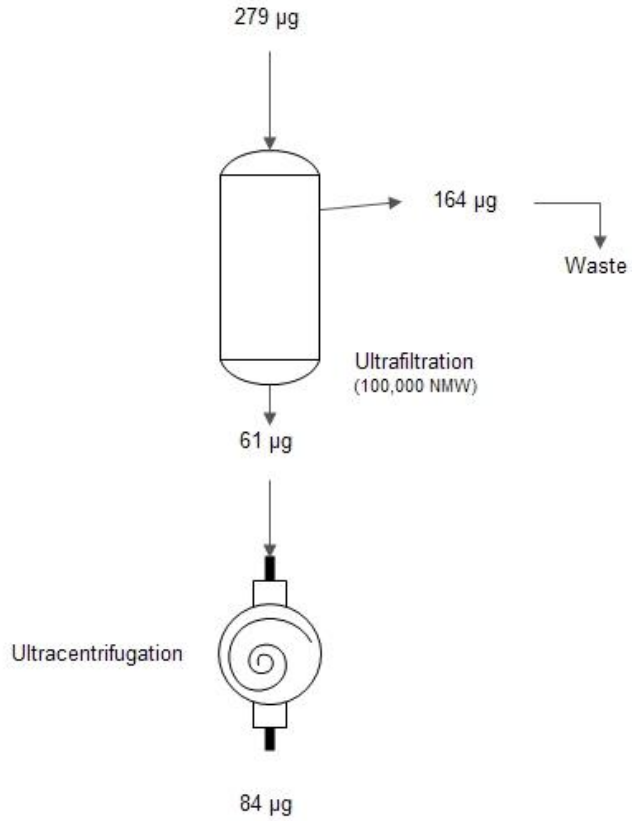

Fig. 4.7- Process diagram representing the ultrafiltration and the ultracentrifugation with the values of total protein in each of the flows.

Besides the concentration of OMVs during the ultrafiltration, it is also relevant to evaluate the protein composition of the samples. In order to quickly evaluate the proteins present in the samples obtained from the ultrafiltration, a 12% SDS-PAGE was performed, thus allowing the evaluation of the protein size present in the collected samples.

Several proteins are present in the different samples analyzed, particularly in the area above the 37kDa, some proteins with low molecular weight can also be observed (Fig. 4.8). Several bands were maintained in the different lanes of the gel. This may be explained by the presence of proteins associated with OMVs, which would not be removed during the ultrafiltration. As expected the final retentate showed the most different proteins profile with several bands disappearing, due to the exclusion of small soluble extracellular proteins by this process.

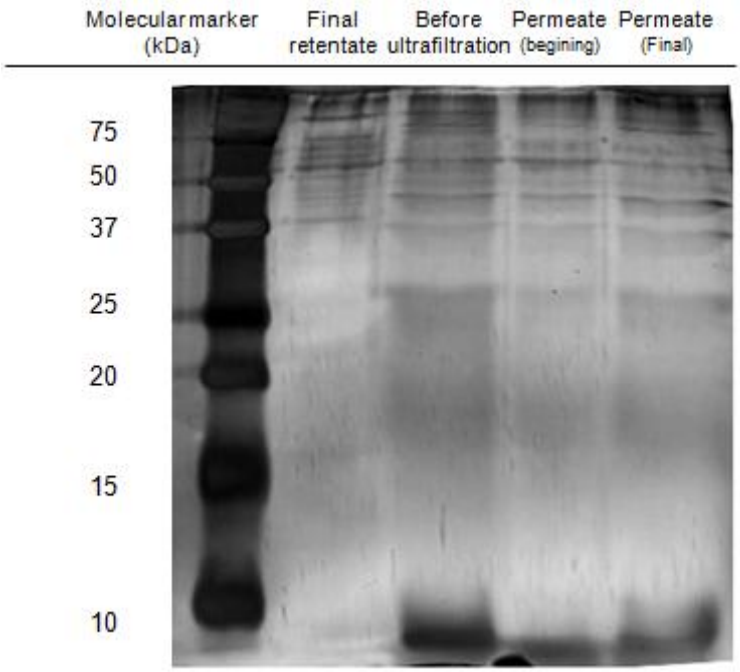

Fig. 4.8- Silver-stained reducing 12% SDS-PAGE using the samples collected from the flows of the ultrafiltration step performed with OMVs from *B. cenocepacia* K56-2 strain.

4.5 Size Exclusion Chromatography of *B. cenocepacia* K56-2 derived OMVs

The results obtained in the proteomic and TEM analysis of *B. cenocepacia* K56-2-derived OMVs showed the presence of contaminants, which inhibited the use of these OMVs for vaccine development without further purification. To increase the purification of the OMV preparations, separation by SEC, using a Sephacryl S-1000 Superfine resin, was employed to increase the purity of OMVs preparations.

The fractionation of the sample by SEC should originate a chromatogram with at least, two different peaks. Due to their larger size, the vesicles should leave the chromatography column first, being followed by cable pilus and flagella, which should leave in a later peak.

It can be observed in Fig. 4.9 a single peak was formed by the OMVs sample injected, however it is also possible to observe a small shoulder on the left side of the peak, which might indicate the presence of OMVs in the injected sample which were not properly separated from the contaminants present, thus occurring overlapping between the peaks.

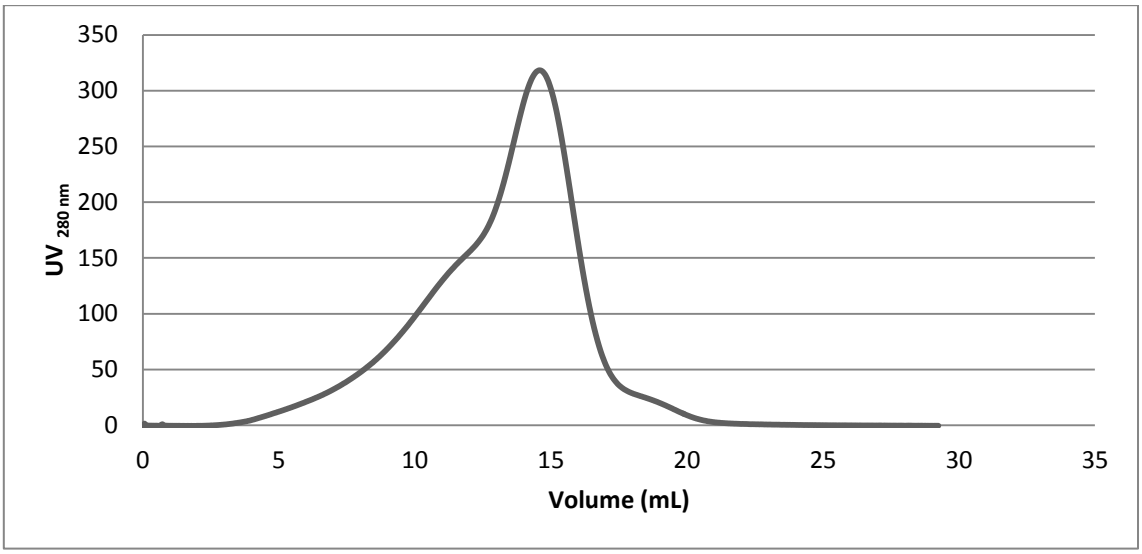

Fig. 4.9- UV absorbance at 280 nm during the SEC of the *B. cenocepacia* K56-2 strain using a 30 cm chromatography column packed with Sephacryl S-1000 Superfine.

Given that the separation of the OMV samples injected in the column were not fully separated from the contaminants, it was raised the hypothesis that the separation wasn't efficiently occurring due to the length of the column (30 cm). In order to test this hypothesis a longer column (60 cm) was used, thus giving more time for the OMVs sample to properly separate during the course of the SEC run.

The use of a longer column did not resulted in complete separation of the two peaks (Fig. 4.10), however the peaks were slightly more separated, yielding a very small peak instead of a shoulder, which indicates a slightly more efficient separation was achieved when compared to the shorter column.

Despite the fact that the desired separation using the longer column was not observed, it was possible to observe an increase in the separation of the peaks by using the longer column, which might indicate that the use of an even longer column would provide better separation.

The use of SEC was not able to produce the desired results, even with the use of columns with different lengths. The increase in the column length appears to have slightly improved separation, however this increase was not enough to achieve the desired result. Since it was not possible to achieve the desired purification using SEC, a different strategy has to be applied in order to obtain purified OMVs, without contamination by cable pilus and flagella.

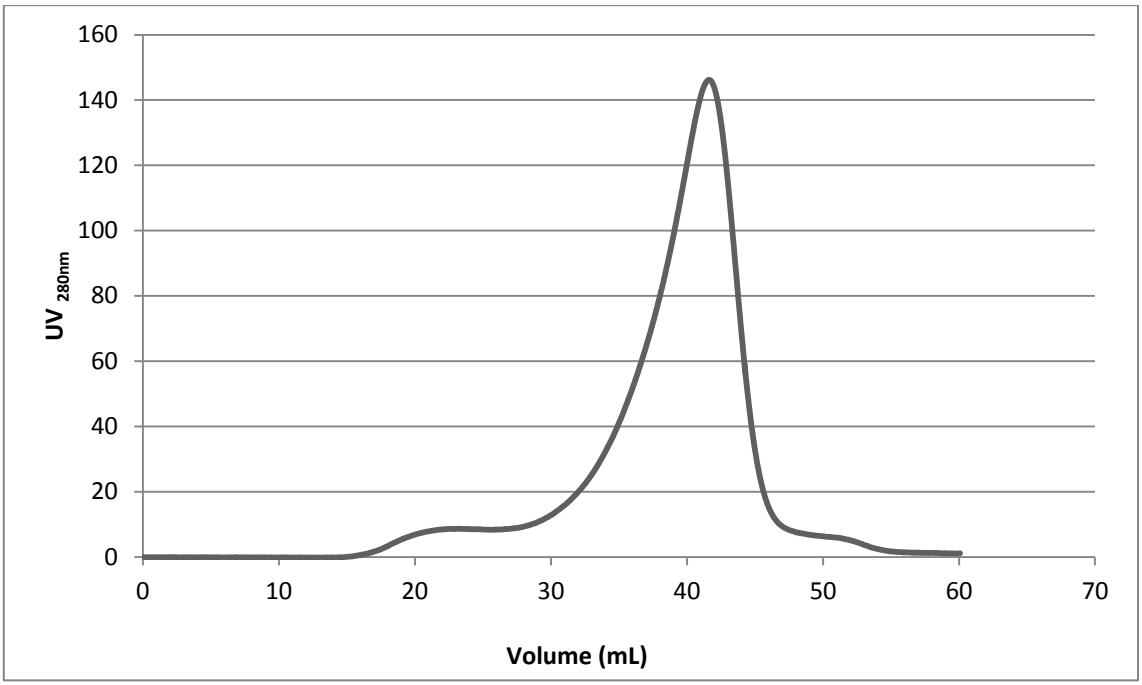

Fig. 4.10- UV absorbance at 280nm during the SEC of K56-2 production using a 60 cm chromatographic column.

4.6 *In vitro* production of OMVs using *B. cenocepacia* AU1054 strain

Since the application of SEC during the purification of *B. cenocepacia* K56-2-derived OMVs didn't exhibited the results expected it was necessary to adopt a new strategy to obtain purified OMVs. Given that the main contaminant of the samples was cable pilus it was decided to use *B. cenocepacia* AU1054 strain which is *cbl*A$^-$, thus not producing cable pilus.

The *in vitro* production of *B. cenocepacia* AU1054-derived OMVs was performed following the same protocol used for *in vitro* production of *B. cenocepacia* K56-2-derived OMVs, using LB medium and maintaining the culture until an OD_{640nm} of 1.5 was reached. The isolation and purification of OMVs was also similar, with the removal of bacterial cells from the extracellular medium, followed by the purification and concentration of the cell-free supernatant. The amount of OMVs obtained after the purification steps was evaluated by quantification of the total protein, which was used as an indicator of OMVs produced. From the initial 2 L of culture it was obtained 0.04 ±0.003 mg of OMVs /L of initial culture.

These productions had the objective of isolating OMVs produced by *B. cenocepacia* AU1054. The samples of purified OMVs were subjected to a TEM analysis in order to evaluate the presence of OMVs and contaminants.

TEM analysis of *B. cenocepacia* AU1054-derived OMVs revealed the presence of OMVs. The diameter of OMVs was usually between 100 and 150 nm, with larger vesicles with diameters between 175 to 225 nm, and smaller vesicles with diameters between 75 to 100 nm were less commonly observed. Cable pilus or flagella were not detected (Fig. 4.11).

The results of TEM analysis lead to the conclusion that the alteration of the strain used for OMV production allowed to obtain purified OMVs.

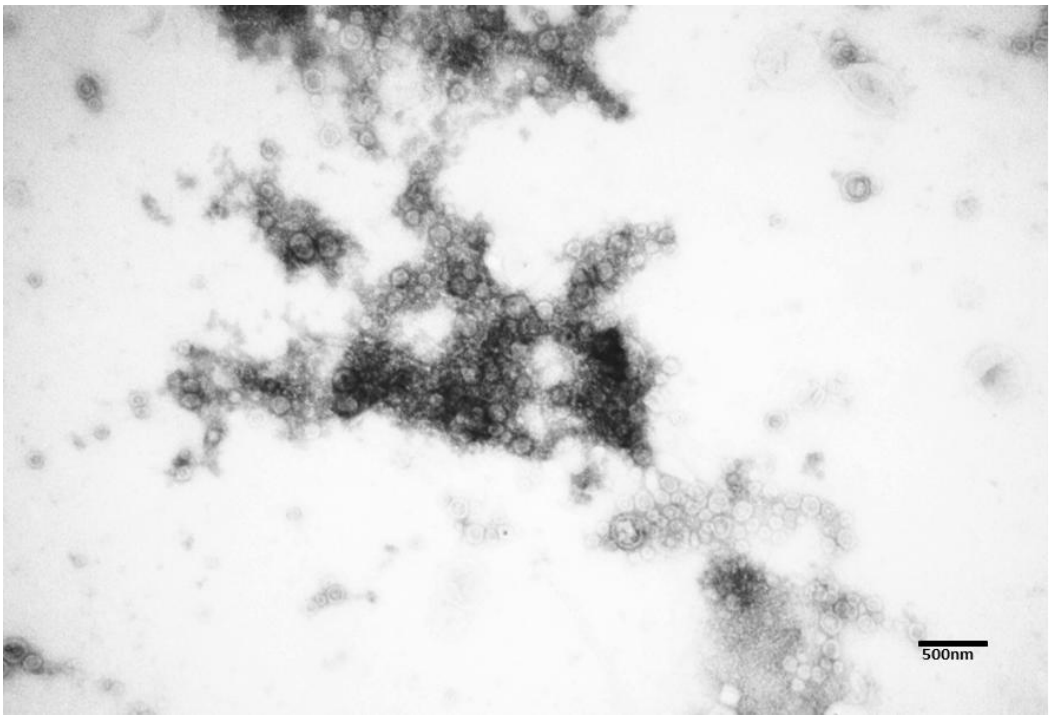

Fig. 4.11- Transmission electron microscopy image from a sample of *B. cenocepacia* AU1054-derived OMVs.

4.7 Downstream processing monitoring of *B. cenocepacia* AU1054-derived OMVs

The culture medium from the culture of *B. cenocepacia* AU1054 was subjected to the same processes of purification which were used in the purification of *B. cenocepacia* K56-2-derived OMVs. The alterations in the compositions of streams during the purification processes was monitored.

The monitoring of the total protein present in different flows of the purification processes was performed following the same protocol used in section 4.4. Recovering the samples in the same points of the process as previously performed.

As expected, the amount of total protein present at the end of the ultrafiltration is lower than at the beginning of the ultrafiltration (Fig. 4.12), which might be explained by the removal of small extracellular proteins present in the cell-free supernatant injected in the ultrafiltration, leaving only larger proteins such as cable pilus, flagella and OMV, in the concentrated OMV sample. The behavior of this strain during ultrafiltration is similar to the behavior *B. cenocepacia* K56-2, with the removal of proteins during the ultrafiltration but not the removal of vesicles.

The OMVs enriched sample exiting the ultrafiltration was subjected to an ultracentrifugation, in order to increase the concentration of OMVs. However, following the ultracentrifugation, concentration of total protein decreased, which was not expected.

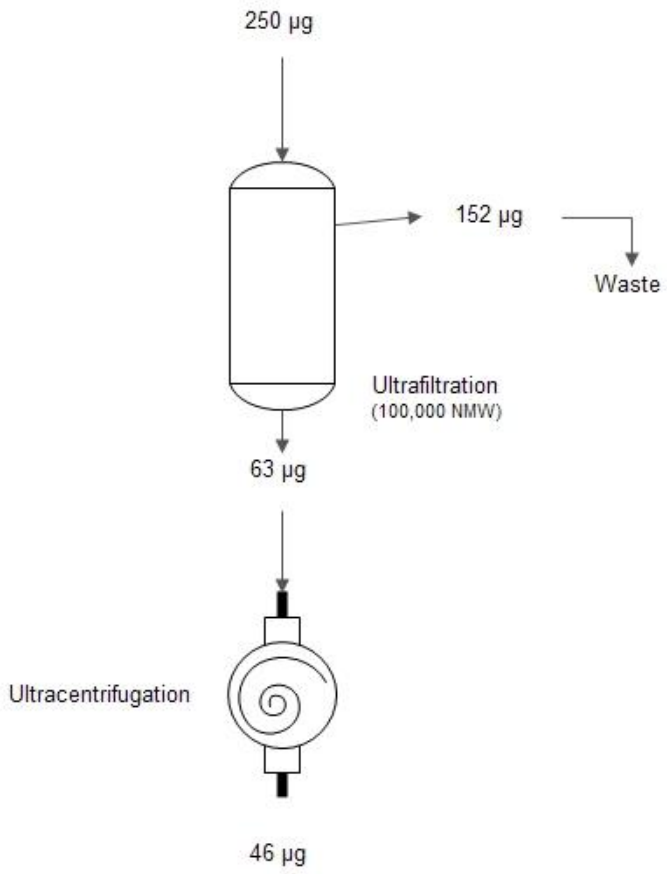

Fig. 4.12- Process diagram representing the ultrafiltration and the ultracentrifugation with the values of total protein in each of the flows.

Samples recovered from the different ultrafiltration streams were also analyzed by SDS-PAGE allowing the identification of the molecular weight of the present proteins.

It can be observed in Fig. 4.13 that one band with a molecular weight between 15 and 20 kDa is present in the beginning of the ultrafiltration and in the permeate but not in the final retentate sample, this suggests that the band might correspond to a protein which was removed from the sample by the ultrafiltration. Several bands can be observed with molecular weights over 25 kDa, these proteins may be associated with OMVs, not being removed during the ultrafiltration.

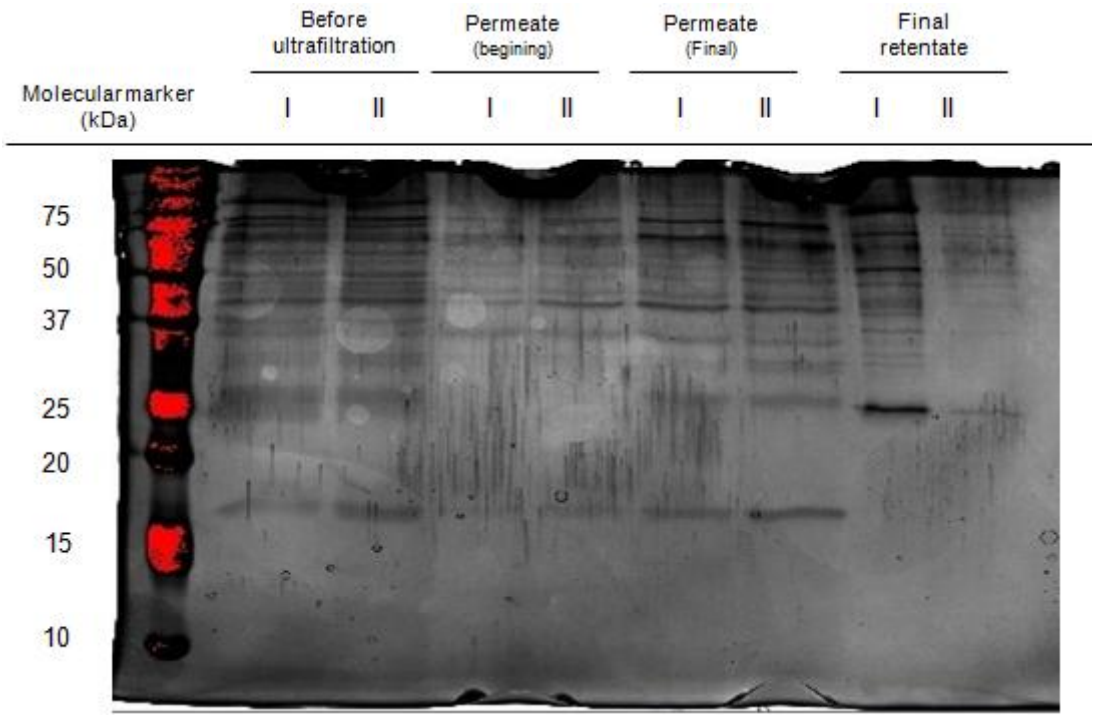

Fig. 4.13- Silver-stained reducing 12% SDS-PAGE using the samples collected from the flows of the ultrafiltration step performed with OMVs from *B. cenocepacia* AU1054 strain. Both the samples are presented in duplicate.

4.8 Cytotoxicity assays

Following the achievement of a purified *B. cenocepacia* AU1054-derived OMVs sample, it was necessary to perform cytotoxic assays to evaluate the possibility of OMVs causing adverse effects on infection models. These assays were performed in two infection models, 16HBE14o- human bronchial epithelial cells and *Galleria mellonella,* thus detecting any possible cytotoxic effects of the OMVs in these models.

4.8.1 Evaluation of *in vitro* cytotoxicity of OMVs

To evaluate possible *in vitro* cytotoxic effects of OMVs, a MTT assay was performed. This assay allows the evaluation of cell survival when exposed to a purified OMV sample. The cells were exposed to increasing amounts of OMVs in order to evaluate the effect of concentration, during a 24 hour period.

Cells exposed to OMVs exhibited a reduction in cell survival (Fig. 4.14), the cells exposed to increasing amounts of OMVs exhibit a dose dependent reduction in the survival. Exposure to 2µg of OMVs resulted in, approximate, 50% of reduction in cell survival.

It was possible to observe from the MTT assays results that 16HBE14o- human bronchial epithelial cells suffered reduced survival after exposure to OMVs during 24 hours, which leads to the conclusion that OMVs might have some cytotoxic effects in this particular *in vitro* model.

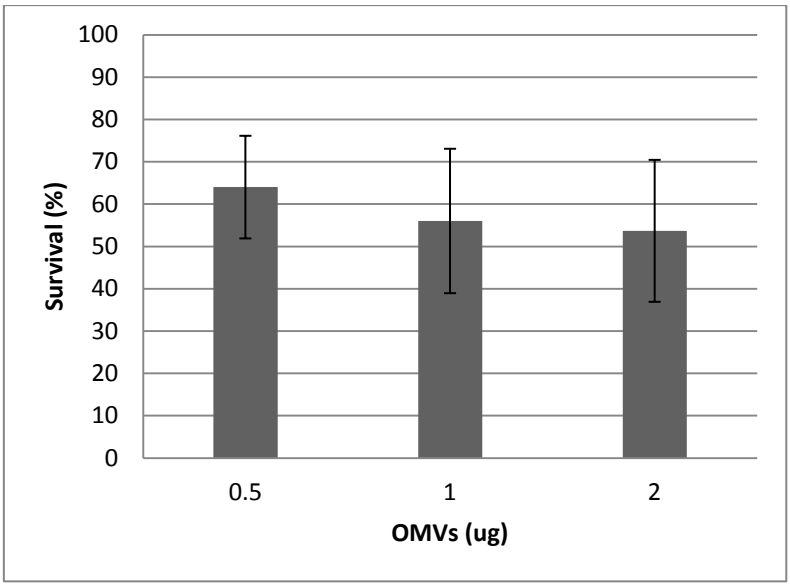

Fig. 4.14- 16HBE14o- cells survival after exposure to purified *B. cenocepacia* AU1054-derived OMVs during a 24 hour period.

4.8.2 Evaluation of in vivo cytotoxic effects of OMVs

The cytotoxic effects of purified *B. cenocepacia* AU1054-derived OMVs were further studied by performing *Galleria mellonella* larvae killing assays, evaluating possible *in vivo* cytotoxic effects.

The larvae were inoculated with a single dose of OMVs, groups of 10 larvae were injected with 0.2 or 1 µg/larvae of OMVs. A control group was injected with of PBS. The survival was monitored during a 96 hours period.

No mortality was observed in the any of the groups injected with OMVs, in both groups all individuals survived until 96 hours after the injection (Table 4.3).

Taking these results into account it is possible to conclude that, contrary to the results obtained in the *in vitro* model, OMVs did not exhibited any cytotoxic effect in the *in vivo* model.

Table 4.3- Number of *Galleria mellonella* larvae alive after a 96 hours period after being injected with a OMV sample isolated from the strain *B. cenocepacia* AU1054

Sample	Larvae inoculated	Larvae alive after 96 hours
PBS	10	10
AU1054 (0.2µg)	10	10
AU1054 (1µg)	10	10

5. Discussion

Several strategies have been employed for vaccine development against Bcc bacteria throughout the years, however the protection achieved by these strategies was not enough [44]. In the last decades, OMVs became attractive candidates as vaccine delivery platforms, due to their immunogenic properties, self-adjuvancy and ability to be taken up by mammalian cells [48]. In fact, it is commercially available an OMVs-based vaccine for *N. meningitidis* and the studies for a *B. pseudomallei* OMVs vaccine are well advanced, but thus far there is no development of an OMVs-based vaccine against Bcc bacteria.

Using the epidemic strain *B. cenocepacia* K56-2 it was possible to produce and isolate OMVs. However, when vesicles purity was assessed by TEM and proteomic analysis, extracellular contaminants such as cable pilus and flagella were present (Fig. 4.2 and Table 4.1). These results suggest that further purification is necessary. Several authors purify the isolated OMVs using density gradients [50; 109], nevertheless this purification process is not easily scalable.

Taking these observations into consideration a SEC step was employed for the purification of isolated OMVs based on a previously described protocol for OMVs purification in *N. meningitidis* [107]. After testing the effectiveness of the SEC process in the purification of the vesicles, it was concluded that the process was not capable of achieving the desired separation, therefore a different strategy was employed.

In addition to the purity evaluation of *B. cenocepacia* K56-2-derived OMVs, the proteomic analysis performed allowed to identify 6 of 15 immunoproteins previously described to be immunogenic across *B. cenocepacia* and *B. multivorans* species and good candidates for vaccine development, namely chaperonin GroEL, elongation factor Tu, OMPA/MotB domain-containing protein, porin, alkyl hidroperoxide reductase/thiol specific antioxidant and phospopyruvate hydratase [122]. These results proven the immunogenic potential of the vesicles. Besides that the 33 proteins identified in the proteomic analysis are commonly found in OMVs derived from other bacteria like *P. aeruginosa* [123], *Neisseria lactamica* [124] and *N. meningitidis* [125].

The OMVs production process was optimized, this optimization was performed by analyzing vesicle production by different Bcc strains, and by optimizing growth conditions. OMVs production was analyzed in several strains from *B. cenocepacia, B. multivorans, B. cepacia* and *B. dolosa*, thus verifying if there was a strain, among the four clinically most relevant Bcc species [10; 119], which could achieve highest OMVs yields. From this analysis it was concluded that K56-2 achieved the best yields, and was used for further optimization of the process.

The culture time is also an important aspect to be considered, since it will affect the amount of OMVs recovered. For the optimization of this parameter two strains were used, namely *B. cenocepacia* K56-2 and *B. multivorans* HI2229, the two most common Bcc species isolated from CF patients. The strain chosen as a representative of *B. multivorans,* was not the strain with best yield of OMVs production, instead a clinical isolate was chosen, since it would be more interesting taking it into account for application of those OMVs in vaccine development. The results obtained showed an

increased in the total protein for longer incubation periods in both strains studied. However the incubation length was maintained, being stopped at late exponential phase. This decision was taken due to the possibility of increase contamination of the sample due to cell lysis. Several other authors also choose to stop the culture at late log phase for the isolation of OMVs [50; 94].

Growth conditions are also a very important factor in OMV production, since they affect vesicle production and characteristics [79; 81]. Taking that observation into consideration, different growth conditions were tested to evaluate the effect in OMVs production. The growth conditions tested in this work were based on the conditions encountered by bacteria in the host's lung, namely osmotic stress and reduced oxygen concentration [121; 126]. Regarding OMVs production under osmotic stress Fulsundar *et al.* have studied the effect of different stresses in vesicle production in *Acineobacter bayly*, among osmotic stress using LB medium supplemented with 500 mM of NaCl, resulting in a small increase in vesiculation [127]. A similar conclusion was achieved by Hood *et al.,* when authors observed an increase of protein secretion in *Acinetobacter baumannii* culture supernatants using LB medium supplemented with 200 mM of NaCl [128]. The results obtained in our study shown a small difference between normal medium and under osmotic stress nevertheless in the opposite sense, with decrease in the OMVs yield in the osmotic stress condition.

Regarding the reduced oxygen availability, Tran and colleagues have evaluated the release of Shiga toxin by a strain of *E. coli*. They evaluated the release of Shiga toxin in microaerobic conditions, and concluded that the release of Shiga toxin was reduced under these conditions [129]. Since Shiga toxin is essentially released incorporated in OMVs [82], it could be inferred that depletion of oxygen may reveal a reduction on OMVs production. A contrary result was obtained in the present study, where the culture of *B. cenocepacia* K56-2 under microaerophilic conditions resulted in an increase of OMVs production. However these results require further experiments to confirmation.

Following the optimization of the OMVs production, it was necessary to develop a strategy which allowed an efficient isolation and purification of OMVs for Bcc vaccination purpose. Initially, the piliated *B. cenocepacia* K56-2 strain was used for OMV production, since a proper purification wasn't achieved it was replaced for the *B. cenocepacia* AU1054 non-piliated strain. OMVs purified from *B. cenocepacia* AU1054 were analyzed by TEM to confirm their purity, and, as expected, they didn't exhibit the presence of cable pilus or flagella. This was an important observation, since it allowed obtaining purified vesicles, without cable pilus or flagella. However The OMV yields obtained in cultures using *B. cenocepacia* AU1054 were lower than using *B. cenocepacia* K56-2, achieving yields of 0.04 ±0.003 and 0.1 ±0.03 mg/L of initial culture, respectively. The OMVs yields obtained in this study were low, Horstman & Kuehn were able to achieve yields between 0.2 to 0.3 mg/L of culture of *E. coli* [94]. Nieves *et al.,* were able to achieve higher OMV yields using *B. pseudomallei*, they were able to achieve 1 to 1.5 mg/L of culture [130].

One interesting possibility to overcome the low OMV yields obtained, is to use molecular engeneering to induce a mutation in the *rpmM* gene, which would lead to a mutant with the capability of over-vesiculate, thus increasing the vesiculation and consequently the OMV yields [85].

The successful purification of OMVs from *B. cenocepacia* AU1054 allowed the realization of a preliminary evaluation of cytotoxic effects in host cells. This evaluation has particular interest since the

final objective of OMVs purification is vaccine development studies. The evaluation of the cytotoxic effects was performed in both *in vitro,* with bronchial epithelial cells, and *in vivo,* with *G. melonella* larvae model. The cytotoxic assays performed *in vitro*, showed a reduction of approximately 50% in cells survival when exposed to OMVs.

The results obtained for the *in vivo* cytotoxic assays revealed no cytotoxic effects to the larvae for the amounts of OMVs used. These differences in the results may be due to the more pronounced susceptibility of the model represented by the cells culture when compared with the insect model. The reduction in cell survival in the *in vitro* cytotoxic assays may be related to LPS, although membrane-bond LPS is known to be approximately 100 times less toxic than free LPS [131] it may still be a source of endotoxicity [48]. Nevertheless, LPS *in vivo* could have an important role of co-adjutancy acting has an immune stimulator of the host immune system being crucial for cellular response of the insect [132].

Cytotoxic studies performed in Caco-2 intestinal epithelial cells, using 100 µg of *Campylobacter jejuni* 11168H-derived OMVs, revealed that the OMVs cause some cytotoxic effects in the cells. Similar results were observed in *G. mellonella* model of infection, the injection with 5 µg or 0.5 µg of *C. jejuni* 11168H-derived OMVs revealed a dose-dependent reduction in the survival of larvae 24 hours postinfection [133]. The results of the cytotoxic assay performed in our study only revealed a dose-dependent reduction in the survival in the 16HBE14o- human bronchial epithelial cells, while in *G. mellonella* no cytotoxic effects were observed. The assays performed in our study used lower amounts of OMVs than the studies performed by Elmi *et al.* which might explain the differences in the results obtained [133].

6. Conclusion and future perspectives

In the future some parts of this work should be repeated in a more in-depth analysis, in order to achieve more solid conclusions. One of the aspects which should be subjected to a more in-depth analysis is the influence of osmotic stress and microaerophilic conditions in the productions of OMVs. Since the reports in the literature regarding this subject are scarce and not in agreement with the results obtained in this study, it would be important to repeat these experiments to confirm the results obtained.

Another aspect which should be analyzed is the cytotoxicity of OMVs. In this study it was only performed a preliminary evaluation. This evaluation noticed, in the *in vitro* model used, that a reduction in cell survival occurred when exposed to OMVs. This reduction may be caused by LPS present in the vesicles, which may be affecting cell survival *in vitro* cytotoxic tests. In the model *G. melonella* the survival was not affected by the exposure to OMVs, which may suggest that *G. melonella* may be able to overcome the possible LPS toxicity.

Performing molecular engineering of the bacteria would allow to achieve over-vesiculation, overcoming the low yields of OMVs production.

In this work it was possible to develop a strategy of production and purification of OMVs from *B. cenocepacia*, using the strain AU1054. This work is a step-forward in the ultimate goal of the project in which it was inserted, which is to investigate the applicability of *B. cenocepacia*-derived OMVs for vaccination purposes.

7. References

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