Production of outer membrane vesicles by the human pathogen *Burkholderia cenocepacia*: purification and preliminary characterization
Rui Filipe Brás Teles Martins

Abstract

Cystic Fibrosis (CF) is an autosomal 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 mellonella*. 16HBE14o- cells suffered reduced survival upon exposure to AU1054-derived OMVs, however this result was not reproduced in *G. mellonella*, with all injected caterpillars surviving the assay.

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

Introduction

Cystic Fibrosis (CF) is an autosomal recessive disease resulting from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) [53], among other problems CF patients suffer from thicker mucus in the lungs [3; 62], which leaves these patients more susceptible to bacterial infections [62; 66]. Among the bacterial infections these patients may suffer it is included infection by species belonging to the *Burkholderia cepacia* complex (Bcc), which despite the small percentage of infections in CF patients being caused by these bacteria [36], leads to the development of problematic infections [21].

Bcc is a group of 20 closely related species of bacteria, to which *Burkholderia cenocepacia*, among other species, belongs [15].

Infection with Bcc bacteria usually causes reduced lung function, and in a subset of patients it may cause necrotizing pneumonia and a rapidly fatal septicaemia termed “Cepacia Syndrome” [21]. The severity of Bcc infection is explained by several characteristics of this group of bacteria, such as the ability of resisting exposure to a wide range of antibiotics [16; 37], ability to form biofilms [12], among others [13; 46; 64], which
facilitate the infection with Bcc strains and difficult eradication of the bacteria. Furthermore, these species are easily spread by contact between infected CF patients [25; 36], saline solutions [14], contaminated hospital equipment [32] and even from the environment [38].

Due to the severity of infections caused in CF patients and the poor outcome of infected patients, as well as the resistance to multiple antibiotics, it becomes interesting to investigate possible alternative treatments and mechanisms to avoid the infection.

There have been several attempts for the development of a vaccine against species of the Bcc, several strategies have been employed in order to achieve this objective [9; 13], with the most promising results being achieved with outer membrane proteins (OMP) [5; 43]. However strategies employed were not able to achieve the desired efficiency.

In the last decade there has been an increasing interest in the use of outer membrane vesicles (OMV) for the development of vaccines, since these vesicles exhibit some characteristics which make them interesting candidates for vaccine development, such as the presence of multiple antigens, being acellular and stability at 4°C [19; 51]. Studies in vaccine development using OMVs have been performed in several bacteria species [2; 27; 50], with one OMV-based vaccine against N. meningitidis already in the market [24] and studies for a vaccine against B. pseudomallei well advanced [47; 49]. However there are no studies regarding development of OMV vaccines against Bcc species.

OMVs are formed by the blebbing of the outer membrane of bacterial cell wall, they do not result from cell death or lysis, and are produced during all phases of growth [7; 44; 69]. OMVs are spherical with a bilayered membrane, with sizes ranging from 50 to 250nm and produced by organisms from the three branches of the tree of life. They are produced by Gram-negative and Gram-positive bacteria [28; 50], Fungi [48], archaea [18], and even in parasites [55], in which seems to be an evolutionary conserved mechanism [35]. These vesicles have several biological functions which provide an advantage to OMV-producing bacteria, they provide resistance to antibiotics [42; 52], provide protection from environmental stresses [44], deliver virulence factors [28; 65], among others [6; 34].

OMVs may be isolated from in vitro cultures. For the isolation of OMVs the most usual process is to perform a bacterial culture until late logarithmic phase, followed by a series of centrifugations and filtrations in order to remove bacteria and concentrate the vesicles [28].

However in order to obtain purified OMVs it is necessary to apply a purification step, usually OMVs are purified by density gradient centrifugation [28]. In order to evaluate the purity of the OMV preparation the method most commonly used is Transmission Electron Microscopy (TEM), which allows to visualize the vesicles and other contaminants, if they are present [28; 41; 44].

This study will evaluate the purity of a B. cenocepacia OMV preparation. Followed by optimization of OMVs production and purification methods. Purified OMVs will be used for cytotoxicity assays, in order to perform a preliminary evaluation of OMV cytotoxicity.

Materials and methods

2.1 Culture conditions and bacterial strains- 10 different bacterial strains were used in this work (Table 1), the strains were maintained frozen in glycerol at -80°C. When necessary strains were defrosted to Luria-Bertani (LB) plates.
OMV production and purification- Vesicles were purified by a method adapted from Huang et al. [29] and Allan et al. [1]. Bacteria were grown in 2 L of LB medium, they were incubated with an initial OD660nm of 0.1 and the culture was stopped when OD660nm reached 1.5. Cells were removed by pelleting (10,000 x g, 30 min.). Supernatants were filtered through a 0.45μm cellulose membrane (Millipore) and concentrated via a 100-kDa tangential filtration concentration unit (GE Healthcare) to approximately 70 mL. The OMVS present in the retentate were pelleted by ultracentrifugation (150,000 x g, 3h), resuspended in 1.5 mL of PBS (pH 7.4). Resuspended vesicles were filtered through a 0.2 μm polyethersulfone membrane (Whatman, GE Healthcare).

Size exclusion chromatography (SEC)- Sephacryl S-1000 Superfine chromatography (GE Healthcare Life Sciences) was performed using an Äkta™ Purifier 10 system, using Tricorn™ 5/100 or Tricorn™ 10/600 columns. 1.5mL of OMV solution were loaded using a 1 mL loop with a flow rate of 0.5 mL/min. The flow-through and elute were continuously collected in 0.5 mL fractions in a FRAC 950 Fraction collector (GE Healthcare).

Optimization of growth parameters, conditions and strains used- In order to optimize the OMVs production process the OMVs production was evaluated in different Bcc isolates. The selected isolates were cultured in 150 mL of LB medium. The initial OD660nm was 0.1 and the culture was maintained until OD660nm reached 1.5. Analysis of OMV production in different points of the growth was performed using the same protocol, differing only in the volume used (500 mL) and the length of the culture (4h, 10h, 16h and OD660nm 1.5).

OMVs production under different growth conditions and mediums was performed using a volume of 500 mL of LB. Two different mediums were used, regular LB medium and LB medium with 300 mM NaCl. Culture was performed until OD660nm reached 1.5, initial OD660nm was 0.1. Microaerophilic conditions were achieved using an anaerobic box (Mitsubishi Gas Chemical Company) and GENbox microaerophilic generator (BioMérieux), bacteria were incubated over-night, until a OD660nm 1.5 was reached. Cells were removed by centrifugation (10,000 x g, 10 min). Supernatants were filtered through a 0.45μm cellulose membrane (Millipore) and concentrated using centrifugal filter Amicon® (Merck Millipore) with 100,000 Da NMWL.

Proteomic analysis- OMVs were analyzed by 2-DE (CBMA- Centre of Molecular Environmental Biology, Department of Biology, University of Minho, Braga, Portugal) using the immobilized pH gradient technique (IPG). OMV samples were resuspended in 7 M urea, 2 M thiourea, 1% (w/v) dodecylmaltoside prior to loading in the first dimension of IPG. IPG strips used were 24 cm non-linear, with pH range from 3 to 10. Isoelectric focusing (IEF) was performed during 3 hours at 7,000 V. The completed first-dimensional strip was subjected to a second dimension using 12% dodecyl sulfate polyacrylamide gel (SDS-PAGE), separation was performed at 110V. Gels were stained with silver nitrate and scanned on a Typhoon Trio laser scanner (GE Healthcare).

Proteins spots were excised manually and identified by mass spectrometry (MS) (Proteomic and Analytical Biochemistry Laboratory, Department of Biology, University of York, York, UK). Proteins in gel spots were digested with trypsin, 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. For identification MS spectra were searched using MASCOT software (Matrix Science) using data from the NCBI non-redundant protein database. 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 [23].

OMVs quantification (BCA assay)- In this study Total protein quantification was used as an indicator of the OMV present in the analyzed sample. Total protein quantification was performed by a bicinchoninic acid (BCA) assay kit (Pierce). Standard solutions of bovine albumin serum (BSA) ranging from 25 to 450 µg/mL.
### Table 1- Characteristics of the Bcc isolates used in this work.

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


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were used to establish a calibration curve. The assays were performed in 96 wells microplates (Orange Scientific) with 200 µL of BCA reagent and 10 µL of sample. Absorbance was read at 562nm in a Spectrostar™ (BMG Labtech) microplate reader.

**MTT assay** - 16HBE14o- human bronchial epithelial cells were maintained in fibronectin/vitrogen coated flasks in Minimum Essential Medium with Earle’s salt (MEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Lonza), 0.292 g/L L-glutamine (Sigma Aldrich) and Penicillin/Streptomycin 100 U/mL (Gibco), in a humidified atmosphere at 37°C and 5% CO₂. Cells were seeded in a 96 well microplate (Orange Scientific) at a density of 7.5x10⁴ cells per well. 0.5 µg, 1 µg and 2 µg of *B. cenocepacia* AU1054-derived OMVs were added to 100 µL of MEM medium and incubated in the same conditions for 24 hours, after which 20 µL of 5 mg/mL [3-(4,5 dimethylthiazol-2-yl)-2,5 tetrazolium bromide)] (MTT) were added in each well and incubated for 3 hours in the same conditions. Following the incubation MTT was removed and 150 µL of 40 mM HCl in isopropanol were added and incubated for 15 min., the crystals were resuspended and plate’s absorbance was read at 590nm in a Spectrostar™ (BMG Labtech) microplate reader.

**Galleria melonella** killing assays - *Galleria melonella* killing assays were based on the methods previously described [45]. A micrometer was adapted to control the volume of a mycrosyringe 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 dishes and incubated in the dark, at 37°C. Survival was followed during a 96 hour period, larvae were considered dead when no response to touch was displayed. Control larvae were injected with PBS (pH 7.4).

**Transmission Electron Microscopy (TEM)** - 10µL of purified OMV preparation were negatively stained by freshly prepared 2% (v/v) uranyl acetate and applied to 200-mesh carbon-coated cooper grids (Electron Microscopy Sciences), being visualized with a JEOL 1200-EX transmission electron microscope.
Results

*B. cenocepacia* K56-2-derived OMVs purity analysis

OMVs were isolated from cultures of *B. cenocepacia* K-56-2 in order to evaluate the purity of the OMVs by TEM. From a 2 L culture of *B. cenocepacia*, it was possible to obtain, following isolation of OMVs, a yield of 0.1 ±0.03 mg of OMVs/L of initial culture. The OMVs obtained were analyzed by TEM, allowing to confirm the presence of OMVs with diameters ranging from 10 nm to 300 nm, with OMVs with 50 nm being more commonly observed (Fig.1). It is also possible to observe the presence of contaminants such as flagella and cable pili (Fig.1).

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

Three different samples of purified OMVs were subjected to a proteomic analysis. 33 different proteins were identified from 73 spots (Fig.2). The proteins identified were divided into 8 different functional groups with the most abundant groups being cell envelope biogenesis and outer membrane, energy conversion and amino acid transport and metabolism. It was possible to observe the presence of a large number of cytoplasmic proteins which is expected since OMVs incorporate proteins from multiple bacterial compartments. It is also possible to observe the presence of cable pilus and flagella, which corroborates TEM results.

A set of proteins identified in the proteomic analysis is of particular interest. This group is composed by proteins which are immunogenic. In this study 6 of 15 immunogenic proteins were identified [54].

Optimization of OMVs production

The process of OMVs production was optimized, by analyzing OMVs production by different strains of the Bcc, under different incubation periods, and under different growth conditions. The strains analyzed were chosen from the four clinically most relevant Bcc species [8; 16]. From the strains tested *B. cenocepacia* K56-2 achieved the best yields (Fig.3).
For the optimization of culture time both *B. cenocepacia* K56-2 and *B. multivorans* HI2229 were tested. The OMVs produced in cultures stopped at different points suggested that longer incubation times lead to increased OMVs production, however the increase in longer incubation times may be due to cell lysis, which was not analyzed in this study.

The final optimization step was the growth conditions. Two stresses, which are present in CF lung, were tested, namely osmotic stress [33] represented by LB medium with 300 mM NaCl, and reduced oxygen concentration [68] represented by microaerophilic conditions.

The growth conditions tested yielded different results, while the use of LB with 300 mM of NaCl resulted in reduced OMVs production compared with LB medium, the use of microaerophilic conditions resulted in an increase of OMV production.

Despite the results obtained in the optimization of OMVs production process, the conditions were maintained. The strains being used was the best producer. The increase in the total protein produced in longer incubation periods may be caused by cell lysis.

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

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>OMV yield (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Broth</strong></td>
<td><strong>Stiring</strong></td>
</tr>
<tr>
<td>LB</td>
<td>250</td>
</tr>
<tr>
<td>0.3M NaCl</td>
<td>250</td>
</tr>
<tr>
<td>LB</td>
<td>60</td>
</tr>
</tbody>
</table>

**Size exclusion chromatography of *B. cenocepacia* K56-2-derived OMVs**

The results obtained in TEM and proteomic analysis of *B. cenocepacia* K56-2-derived OMVs suggested that further purification of the samples was required.

To further purify the OMVs a SEC step was applied to the samples, to increase purity of the preparation.

The OMVs were loaded into a Tricorn™ 5/100 with 30 cm of length. The use of this column did not allow complete separation of the OMVs and the contaminants present in the sample, with only a partial separation being achieved.

In order to increase the separation between the OMVs and the contaminants in the sample, a longer column was used. The reasoning behind the employment of a longer column was that the increased retention time in the column would allow for a more complete separation.

The longer column used was a Tricorn™ 10/600 with 60 cm of length. OMVs were also loaded into the column. The separation of the samples was greater using the longer column, however complete separation was not achieved, with an small overlap between the OMVs fractions and contaminants fraction, thus not achieving complete separation between the samples and contaminants.
Production of *B. cenocepacia AU 1054*-derived OMVs

Since the use of SEC for the purification of *B. cenocepacia K56-2*-derived OMVs did not achieved the desired results, a different strategy was employed for the achievement of purified OMVs.

Taking into consideration the observations from the TEM analysis of *B. cenocepacia K56-2*-derived OMVs, in which Cable pili and flagella were the main contaminants, we reasoned that the use of a strain not capable of producing cable pili would reduce the contamination of the OMV preparation.

To achieve this objective the strain used for OMVs production was switched to *B. cenocepacia AU 1054*, which is CblA-, thus not being able to produce cable pili. This strain was used for production of OMVs, the protocol used for production and isolation of *B. cenocepacia AU1054*-derived OMVs was the same as the one used for *B. cenocepacia K56-2*. From the 2 L of initial culture and following the isolation of OMVs from *B. cenocepacia AU 1054*-derived OMVs, it was possible to obtain 0.04 ±0.003 mg of OMV/L of initial culture.

**Transmission Electron Microscopy of *B. cenocepacia AU1054*-derived OMVs**

*B. cenocepacia AU1054*-derived OMVs were analyzed by TEM to confirm the presence of vesicles and evaluate the purity of the OMVs.

TEM analysis of OMVs isolated from *B. cenocepacia AU1054* revealed the presence of OMVs with diameters between 100 and 150 nm. It was also possible to observe vesicles with smaller diameters (75 to 100nm) and large diameters (175 to 225 nm) were less commonly observed (Fig. 3).

It is also important to notice that cable pilus or flagella were not observed in these preparations (Fig.4).

**In vitro cytotoxic effects of *B. cenocepacia AU1054*-derived OMVs**

Following the isolation of purified OMVs from *B. cenocepacia AU1054* it became possible to perform a preliminary evaluation of cytotoxic potential of OMVs. Initial cytotoxic assays were performed *in vitro* by performing MTT assays in 16HBE14o- human bronchial epithelial cells. Exposure to OMVs revealed a reduction in cell survival, which increased with an increase in the amount of OMVs to which cells were exposed, reaching almost 50% in the highest quantity of OMVs (Fig. 5).

![Fig. 4](image)

**Fig. 4**- Transmission electron microscopy image from a sample of *B. cenocepacia AU1054*-derived OMVs.

![Fig. 5](image)

**Fig. 5**- Cell survival after exposure to purified *B. cenocepacia AU1054*-derived OMVs during a 24 hour period.
In vivo cytotoxic effects of B. cenocepacia AU1054-derived OMVs

Following the in vitro cytotoxic assays performed with OMVs, the cytotoxic effects of OMVs were also tested in Galleria melonella. The results obtained from the in vivo testing of cytotoxic effects were different from the MTT assays. Exposure to purified OMVs in G. melonella did not result in reduced survival, in fact all larvae injected with OMVs survived the testing period (Table 3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Caterpillars inoculated</th>
<th>Caterpillars alive after 96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>AU1054 (0.2µg)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>AU1054 (1µg)</td>
<td>10</td>
<td>10</td>
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</table>

Table 3- Number of Galleria mellonella caterpillars alive after a 96 hours period after being injected with a OMV sample isolated from the strain B. cenocepacia AU1054.

Discussion

The development of a vaccine against Bcc species has been subject to various studies, however they were not able to achieve enough protection [13].

Recently OMVs have been employed in the development of vaccines due to their characteristics [51]. Using B. cenocepacia K56-2 we were able to produce and isolate OMVs. Assessment of purity of isolated OMVs by TEM and proteomic analysis revealed the presence of contaminants, requiring further purification. Several authors use density gradients for OMV purification [4; 63], however this purification process is not easily scalable.

Taking those observations into consideration we employed a size exclusion chromatography step for the purification of isolated OMVs based on a previously described protocol for OMVs purification in N. meningitidis [59]. Testing of SEC efficiency in the purification of OMVs revealed that the process was not capable of achieving the desired results, therefore a different strategy was employed.

As said before the proteomic analysis of B.cenocepacia K56-2-derived OMVs revealed the presence of 6 of 15 immunoproteins across B. cenocepacia and B. multivorans species, which are good candidates for vaccine development, namely chaperonin GroEL, elongation factor Tu, OMPA/MotB domain-containing protein, porin, alkyl hydroperoxide reductase/Thiol specific antioxidant and phosphopyruvate hydratase [54].

Besides, the 33 proteins identified in the proteomic analysis may also be encountered in OMVs derived from other organisms, such as P. aeruginosa [11], N. lactamica [60] and N. meningitidis [67].

In the optimization of the OMVs production process, when optimizing culture length a B. multivorans strain was used, this strain is a clinical isolate, and was used in order to have a representative of the two clinically relevant species. The results suggested that using longer incubation periods lead to increased OMVs yields, however the increase in OMVs production may be caused by cell lysis. Since the cell lysis was not evaluated, the incubation length was maintained, this incubation period is similar to the period used by other authors, which stop the cultures at lat log phase/early stationary [4; 28].

Growth conditions have an important effect in vesicle production [40; 41]. Taking that observation into consideration it was necessary to evaluate the effect of growth conditions in OMV production. Two different conditions were tested, osmotic stress and microaerophilic conditions.
conditions, which, as previously stated, represent the conditions encountered by bacteria in host’s lungs. Literature reports regarding OMV production under osmotic stress and microaerophilic conditions are scarce, however some reports may be found. Regarding OMV production under osmotic stress Fulsundar et al. have studied the effect of different stresses in vesicle production in Acineobacter bayly, concluding that LB medium supplemented with 500 mM of NaCl for the culture of A. bayly, resulted in a small increase in vesiculation [22]. The results obtained in the present study are not in agreement with the results obtained by Fulsundar et al. In respect to reduced oxygen availability, Tran et al., 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 [56]. Since Shiga toxin is released by OMVs [17], it may be possible that in E. coli strain used also releases Shiga toxin via OMVs. The results obtained in the present study, once again, are not in agreement with the literature, having achieved higher OMV yields in microaerophilic conditions.

For the development of an alternative purification strategy, and taking into account the results obtained in TEM and proteomic analysis of B. cenocepacia K56-2, the strain used for OMVs production was replaced with B. cenocepacia AU 1054, which does not produce cable pilus or flagella, facilitating the purification of OMVs.

The yields obtained by B. cenocepacia AU 1054 are lower than the yields obtained by B. cenocepacia K56-2, however TEM analysis revealed the presence of OMVs in B. cenocepacia AU 1054 OMVs preparations, without the presence of contaminants, thus achieving the objective of producing purified OMV preparations using an alternative strategy.

The evaluation of cytotoxic effects both in vitro and in vivo revealed some differences, while the in vitro model exhibited reduced cell survival, the in vivo model did not exhibited reduced survival. This discrepancy between the models used may be explained by the lipopolyssacharide (LPS) toxicity, which may cause some toxicity [51]. Work performed by Elmi et al., revealed that Campylobacter jejuni 11168H-derived OMVs revealed dose-dependent cytotoxic effects in Caco-2 intestinal epithelial cells and in G. mellonella [20], while in this work only in 16HBE14O- human bronchial epithelial cells was possible to observe cytotoxic effects derived from OMVs exposure, which may be explained by the higher OMVs dose used in the work performed by Elmi et al [20].

Conclusions and future work

Some aspects of this work should be further investigated. The effect of osmotic stress and microaerophilic conditions should be further studied, repeating the assays performed, in order to achieve a more solid conclusion, since literature reports are not in agreement with the results obtained.

The cytotoxic effects of OMVs should also be subjected to a more in-depth analysis, evaluating the effect of exposure to higher amounts of OMVs.

One interesting strategy which could be used in the future to overcome the low OMVs yields obtained is the use of genetic engineering techniques to cause a mutation in the rmpM gene, which would lead to a mutant with increased vesiculation [57], thus increasing the OMVs yields.
This work lead to the development of a strategy to obtain purified OMVs from *B. cenocepacia*. This strategy allows the production of OMVs suitable for the development of future studies in the development of OMVs-based vaccines.

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**References**


