# Function Analysis of Choline Binding Domains of LytA, LytC and CbpD in The Biofilm Formation Process of Streptococcus Pneumoniae

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**Research Article**

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# Abstract

**Background**: Choline binding proteins (CBPs) are a family of proteins that can interact with pneumococcal cell wall by choline binding domains (CBDs). In this study, we found a modified choline binding repeat (ChBp-I) with a pI of 7.66 can promote the development of biofilm *in vitro*. Thus, we aim to characterize the function of CBDs of LytA, LytC and CbpD in biofilm formation.

**Results**: By transcriptome analysis, 81 genes were identified as down regulated and 138 genes were up regulated (|log2 fold change|≥1.5) under ChBp-I of 50mg/L. The up regulated genes are well clustered in membrane transport (carbohydrate, lipid, protein, cation and phosphate) and carbohydrate metabolism (fructose, mannose, galactose, starch, sucrose, amino sugar and nucleotide) related pathways. The up-regulated genes are mostly regulated the same under CBD-A, CBD-C and CBD-D. Phenotype analysis reveal high concentrations of CBD-C and CBD-D (>100μg/mL) but not CBD-A (negative charged) can promote the biofilm formation. Meanwhile, the existence of CBD-C and CBD-D promote the growth rate and both CBDs inhibit the autolysis of pneumococcal cell. By component analysis, these three CBDs were proved involved in the regulation of extracellular DNA, protein, cation and phosphate, and promote the forming of insoluble precipitates.

**Conclusions:** The binding of CBPs can influence the membrane transport pathways and react with extracellular DNA and protein to promote biofilm formation in *S. pneumoniae*.


# Background

*Streptococcus pneumoniae* is a Gram-positive bacteria, that usually colonizes the upper respiratory tract [1]. It can cause invasive disease like pneumonia, sepsis, and meningitis [2]. As an important human pathogen, the gene regulation and pathogenesis of *S. pneumoniae* have been conspicuous and inclusive investigated in these years. One of the features of pneumococcal cells is the phosphorylcholine (P-Cho) modified teichoic acid (TA) on the cell wall and membrane. The P-Cho on the cell wall is essential for the interaction G-protein-coupled platelet-activating factor receptor, which is important for the colonization in the upper respiratory tract [3]. Meanwhile, P-Cho is also a binding site for the self-encoded choline binding proteins (CBPs). CBPs play important roles in the pathogenesis of *S. pneumoniae* [4].

The CBPs in *S. pneumoniae* are composed by a function domain and a choline binding domain (CBD) [5]. The CBDs contain numerous homologous choline binding repeats (CBRs). The binding activity of CBRs is important to the functions of CBPs [6]. Among the CBPs, the function of LytA, LytB, LytC and CbpD that can be detected in extracellular of *S. pneumoniae* have been well studied. LytA and LytC play roles in autolysis during the stationary phase and fratricide during competence process [7]; LytB play roles in cell separation during division [8]; CbpD is a murein hydrolase, it play roles in fratricide together with LytA and LytC [9]. During logarithmic growth phase pneumococcal cells is protected from LytA, and it prefers to bind the division sites [10]. In LytC, the conformation CBRs is hook-shaped, this make it can only hydrolyze non–cross-linked peptidoglycan chains [11]. Due to the difference of sequences and

conformations CBPs exhibit with different binding properties, this is also related to their natural functions.

On the other hand, the functions of autolysis related CBPs are well regulated in logarithmic phase. For the lack of signal peptide, the newly synthetized LytA is mainly localized in the cytoplasm. Extracellular LytA, together with LytB and LytC are mainly located on the septum of the dividing cells, and this binding is not harmful to pneumococcal cells [6]. The increase of extracellular LytA mainly occurs at the stationary phase, which may relate to cell wall degradation [6]. The expression level of LytA and CbpD are both low at logarithmic phase. But the expression level of LytA and CbpD can be activated by late competence genes [12]. CbpD can digest the peptide cross-linkers of the peptidoglycan, which provides more accessible substrates for LytC [11]. Besides at stationary phase, the ComDE pathway was also up regulated when encountered with immune pressure, antibiotic and other growth Inhibition factors [12]. At both these time points massive LytC, LytA and CbpD can bind to pneumococcal cell wall.

Both the functions of LytC, LytA and CbpD have been well characterized with the obvious functional module. But the functions of the choline binding domains (CBDs) are largely unknown besides binding to the cell wall. It has been reported that pneumococcal cells lacking CBPs are more hydrophobic and exhibit with poor adherent [13]. Meanwhile, mutants lacking LytA, LytC, LytB, CbpA, PcpA and PspA both had a decreased capacity to form biofilms [14]. Cation mediate binding with DNA was detected to several CBPs, and this binding was supposed important for biofilm formation, for LytB-DNA complex was detected in the biofilm [15]. Beside cation and DNA, extracellular protein was also required for biofilm formation [16]. In the present study, an increased biofilm formation was detected with the existence of ChBp-I a choline binding repeat (CBR) derived from LytA. To clarify the functions of extracellular CBDs, we detected the role of CBDs of LytC, LytA and CbpD in gene and growth regulations. The results showed these CBDs paly roles in carbohydrate metabolism and membrane transport of extracellular phosphate, cation, DNA and proteins. These data are helpful in understanding of the putative role of LytC, LytA and CbpD in biofilms formation besides inducing autolysis.

# Results

### ChBp-I2 enhance biofilm formation of *S. pneumoniae in vitro*

In previous studies, we reported the binding capacity of LytA-derived choline binding peptides (ChBp) to pneumococcal cells. Similar as the choline binding domains in CBP, ChBps are supposed with stronger binding capacity to choline residues on the cell surface for they get smaller spatial obstacles. During the analysis of ChBp-I2 we found it can promote the biofilm formation of *S. pneumoniae in vitro* on a dose dependent manner (Fig.1). And this promotion ability is much stronger than the two rest homologous sequence (ChBp-I1 and ChBp-I3). After analysis of these three peptides, we found ChBp-I2 got a higher isoelectric point (7.66) than the rest twos (7 and 5.09). Considering the biofilm formation promotion activity of ChBp-I2, we further analyzed the content of extracellular CBPs during the growth of *S. pneumoniae in vitro*. As a result, the choline binding proteins and high molecular weight protein were only

detected during the stationary phase. Low molecular weight proteins (<10Kd) that can be enriched by DEAE were detected at both time points (Fig.2). These proteins may derive from the medium for the proteins are enriched at 2th hour, when the cell dense is low. These indicate the content of extracellular CBPs is low during the logarithmic phase, and extracellular CBPs may mainly function at late logarithmic phase and stationary phase.

### ChBp-I2 influences membrane transport carbohydrate metabolism pathways

Beside biofilm formation promotion, we found ChBp-I2 can enhance the growth of pneumococcal cells with a small extent at the initial culture period (not shown). Thus, we infer ChBp-I2 may influence the gene expression of *S. pneumoniae*. After analysis, a total of 81 genes were identified as down regulated and 138 genes were up regulated (|log2 fold change|≥1.5). The differentially expressed genes are mainly enriched in membrane transport and carbohydrate metabolism related pathways. The membrane transport pathways include carbohydrate, lipid, protein, cation and phosphate. Meanwhile, the carbohydrate metabolism pathways include fructose, mannose, galactose, starch, sucrose, amino sugar and nucleotide sugar metabolism (Figure 3). By Q-PCR we found the regulations of these genes are mostly dose dependent (Fig.S2). Considering the only binding activity of ChBp-I2, we infer the regulations of these DE genes may relate to the physical changes on cells surface. Considering the increased extracellular CBPs at late logarithmic phase and stationary phase, the DE genes were analyzed to genes regulations at late logarithmic phase to analyze the potential regulations of extracellular CBPs. There are 12 genes were co-up regulated and 37 genes were regulated conversely. 45 genes were co-down regulated and 21 were regulated conversely (Figure 4). This indicates most of these DE genes are down regulated at late logarithmic phase, which may relate to the decrease of growth rate caused by nutrition exhaustion.

### CBDs function differently on growth, biofilm formation and gene expression of *S. pneumoniae*

According to gene expressions at late logarithmic phase, the expression of LytA, LytC and CbpD were characterized as up-regulated. Thus we further analyzed some growth phenotype under the CBDs of these three proteins. When culture in mediums supplemented with the CBDs of different concentrations, CBD-A and CBD-D of high concentration (200μg/mL) exhibits a certain inhibitory effect on growth rate. Similar with ChBp, both of three CBDs can promote the growth of *S. pneumoniae* at different degrees, the optimum concentrations are both about 25μg/mL, which is lower than ChBp-I2 (100μg/mL) but higher than ChBp (3μg/mL) (Figure 5A). Meanwhile, CBD-C and CBD-D of high concentrations (100-200μg/mL) can promote the formation of biofilm (Figure 5B). Through phenotypic observation, we found the cells under CBD-C are easy to form aggregation when place at room temperature, and both CBD-A, CBD-C and CBD-D show inhibition to autolysis. Under microscope the biofilm under CBD-C form large area of membranous region, which is different to the ones with CBD-A and CBD-D (Figure 6, Figure S3). By PI analysis we found the CBRs in LytA are tending to be negative charged, the CBRs in CbpD are positive charged under neutral environment. CBD-C contains similar number of negative charged and positive charged CBRs (not shown). A higher PI may be helpful in in explaining the biofilm formation promotion activities of CBD-C and CBD-D.

### CBDs are involved in the regulation of extracellular nucleic acid and protein

For the similar phenotypes of pneumococcal cells under CBDs and ChBp-I2, we further analyzed the expression of DE genes (12 down regulated and 14 up regulated genes) under the existence of CBDs. After analysis we found most of the DE genes are up-regulated under CBDs, indicating the CBDs may functions differently (Figure S2). To further define the function of extracellular CBDs, four kind of components were tested (cation, phosphate, protein and nucleic acid) in the culture medium which related to the regulated pathways. As indicated, the most significant feature is the increased extracellular protein. After ultrafiltration concentration, the proteins under CBD-A, CBD-D and ChBp are more than 4mg/mL, the proteins in the control group is only about 0.5mg/mL (Figure 7A). When being precipitated by TCA, the high molecular weight proteins (HMWp) were mainly detected as insoluble form in the mediums of CBD-A and CBD-D (Figure S4). Meanwhile, the extracellular nucleic acid is increased with CBD-A, but decreased under CBD-C and CBD-D (Figure 7B). A slight increase in extracellular phosphate (CBD-C and ChBp) and cation (CBD-A and CBD-D) was detected (Figure 7C, 7D). Taken together, CBD-A is supposed to promote extracellular transport of nucleic acid participate in the formation of HMWp. CBD-C can increase the concentration of extracellular phosphate and decrease extracellular nucleic acid. CBD-D can promote the formation of HMWp, decrease extracellular nucleic acid, and increase extracellular cation.

## Discussion

Biofilm formation is not only involved in colonization but also important for the survival of pneumococcal cells *in vivo* [1], the cells in biofilm exhibit an inherent tolerance to antibiotic therapies and host immune attack [17]. The binding of CBPs were shown contribute to S. pneumoniae biofilm formation, for the cells with CBPs mutations or lack of choline residues in the cell wall both show reduction in biofilm formation [14]. However, mutants of CbpD did not show decreased capacity to form biofilms in vitro. These may relate to the low expression level of CbpD at logarithmic phase [18]. The present of CBPs on the cell wall correlate with weakened hydrophobic and electronegative net surface charge, which may facilitate biofilm formation and adherence to host cells [13, 19]. Both the biofilm formation and surface charge capacities are more distinct in non-encapsulated strains [13]. A reduction of capsule expression is detected when contact with human epithelial cells, which may correlate with stable adhesion and biofilm formation [20]. As indicated in the present study CBR with pI about 7 is more conductive to biofilm formation, whereas the pIs of the extracellular CBPs are different indicating complex conditions *in vivo*. The binding of CBPs to the cell was considered as the initial step of biofilm formation, for the biofilms formed in the presence of a high choline concentration were not tightly attached [14]. Besides displayed on the cell wall, CBPs was supposed being consisted in the intercellular matrix of biofilm, for biofilms formed by a LytA or LytC mutant were thinner [14].

To our knowledge there is no study describes the gene alternations mediated by the binding of CBPs. The DE genes displayed in the present study was supposed being regulated by surface properties changes post the binding of CBPs. In *S. pneumoniae*, the carbohydrates transport pathways are

controlled by the transcriptional regulator CcpA. The present of carbohydrate like glucose repressed the pathways of non-favourite carbon source. Thus, the up regulation of carbohydrate metabolism pathways described here may relate to the limitation of intracellular glucose or changes in of cell wall permeability. Several of these carbohydrate metabolism pathways have been proved up regulated during the biofilm process such as fructose, and galactose [21, 22]. Meanwhile, the increased extracellular DNA in CBD-A group and increased extracellular protein in CBD-A, CBD-D and ChBp groups may also correlate with the binding and regulation of membrane transport pathways. DNA release in pneumococcal cells was reported as autolysin dependent, mutants of LytC, LytA and CbpD are both attenuated in DNA release [23, 24]. As indicate here we infer the binding of CBD-A can promote DNA release (Fig). Increased proteins synthesis de novo was found during the biofilm development, and the proteins involved in virulence, adhesion and resistance were up regulated. Through no significant changes in the virulence related proteins was detected, the increased genes involve in nucleotide metabolism and translation reveal an increasing in protein expression [25]. Besides, the reaction between certain CBRs with phosphate may also play roles in the complex structure of biofilm [26].

Positively charged amino acid sequence in LytB and LytC have been proved with DNA binding capacity [27]. In LytC the positively charged amino acid in the function domain and choline binding domain were considered with no DNA binding capacity, for the binding of DNA did not influence the function and binding of LytC [27]. As we suppose the CBD-C can interact with extracellular DNA, the DNA and choline residues may bind at different sites for choline mainly bind at the hydrophobic interface [28]. One evidence support this hypothesis is the present of CbpF inhibited the reaction of LytC and DNA, and CbpF mainly influence the function of the CBD of LytC [27, 29]. Besides, extracellular DNA was also react with other polymeric substances like polysaccharides, other proteins and metabolites; extracellular DNA was also required for the initial attachment to human cell and abiotic surface [16, 17]. All the interactions between these substances may form insoluble structures, resulting the decrease of extracellular DNA and protein (Figure 7). In conclusion, the CBDs of LytC, LytA and CbpD were proved influencing the membrane transport and play roles in biofilm formation by reacting with extracellular substances.

# Materials And Methods

### Bacterial strains and growth conditions

The main *S. pneumoniae* strain used in this study is ATCC49619, two isolated strains SWU10 and SWU11 were also applied in the biofilm formation assay of ChBp-I2. Routinely, pneumococcal strains were growth in brain heart infusion (BHI) both supplemented with 5% newborn calf serum for growth and biofilm formation assay.

### Peptides synthesis and purification of recombine choline binding domains

The two peptides (ChBp and ChBp-I,) were synthesized as described previous (Table S1) [5]. The sequences of choline binding domains (CBDs) of LytA, LytC and CbpD were amplified by corresponding primers with NcoI and HindIII modifies (Table S2). The sequences were then cloned to pET28a, and then

the recombinant pET28a was transferred to BL21 (DE3). The BL21 (DE3) cells were induced by isopropyl-b-D-thiogalactopryanoside (IPTG) in LB broth under different conditions at an initial concentration about 0.6 (OD595). After induction, the cells were collected by centrifugation at 8000 g for 5 min and re-suspended in TM buffer (50 mM Tris-maleate, pH 6.9). After freeze-thaw and ultrasonic the supernatants were transferred to a new tube post concentration at 8000 g for 10 min. The CBDs in the supernatants were purified by DEAE Sefinose, washed with TM buffer containing 1.5 M NaCl and eluted with TM buffer plus 1.5 M NaCl containing 2% choline (8 times column volume). All the eluates were detected by SDS-PAGE (Figure S1).

### Transcriptome sequencing and quantitative reverse transcription PCR

ATCC49619 cells of were cultured to a concentration about 0.1 (OD595). Then the medium was divided into two parts at equal volume, one of which was supplemented with ChBp-I of 50μg/mL. Two hours post incubation at 37°C the culture mediums were removed by concentration at 5000 g for 5 min at 4°C. Then the precipitates were collected and transported on dry ice to Majorbio (Shanghai, China) for transcriptome sequencing. The sequencing data were applied for gene expression analysis by RSEM and DEGseq. The genes were considered as differentially expressed (DE) with |log2 fold change|≥1.5 between these two groups. The functions of the DE genes were analyzed with EggNOG database.

To validate the expression of DE genes, 26 pair primers were designed and applied in q-RT-PCR (Table S3). In brief, pneumococcal cells were culture to about 0.1 (OD595) in normal BHI. Then the cells were transferred to mediums (1:4 dilution) containing ChBp-I of 100 and 50μg/mL with three repetitions. Two hour post incubation at 37°C the cells were harvested by concentration at 5000 g for 3 min at 4°C. RNAs of these cells were extracted with RNAiso Plus (TaKaRa, China), and reverse transcripted with TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, China). The qPCR were conducted with PerfecrStart Green qPCR SuperMix (TransGen Biotech, China). In addition, the qPCR were also applied to CBD-A (LytA), CBD-C (LytC) and CBD-D (CbpD) of 200 and 100μg/mL with the same procedure.

### Growth and adhesion under CBDs

ATCC49619 cells of were cultured to a concentration about 0.1 (OD595). Then the cells were 1: 10 diluted and added to BHI mediums containing ChBp-I2 (200, 100, 50, 25, 12.5, 6, 3μg/mL) at equal volume, then all the volumes were transferred to 96 well plate with three replicates of 200 μl per well. The plate was incubated at 37°C, the plate was tested by microplate reader every hour post incubation. At 6h post incubation the mediums were discarded. The wells were then stained with 0.1% crystal violet (50μl) for15min. After five washes with normal saline the remaining crystal violet was dissolved with anhydrous ethanol (100μl) and detected by microplate reader (OD595). The growth and adhesion assay was further conducted to CBD-A, CBD-C, CBD-D (Serial diluted with an initial concentration of 100μg/mL) and ChBp (Serial diluted with an initial concentration of 25μg/mL). After stained with crystal violet the attached cells on base of 96-well of these three groups were captured under microscope, and then the cells were dissolved with anhydrous ethanol and detected as above.

### Components changes in culture medium

Logarithmic ATCC49619 cells (OD595≈0.1) was 1:5 diluted in fresh BHI mediums supplemented with CBD-A (100μg/mL), CBD-C (100μg/mL), CBD-D (100μg/mL), ChBp-I2 (50μg/mL) and ChBp (12.5μg/mL) respectively, using normal BHI as control. All the volumes were further incubated at 37°C for 2h, then the cells were harvested by centrifugation at 5000 g for 5 min, re-suspended in phosphate buffer (10mM) and placing at room temperature for 10h. The supernatants were divided into four parts. 2mL supernatants of each group were mixed with sodium hydroxide (0.1 g/ml, 200μL) with three repetitions; the precipitation of divalent salt was collected by centrifugation at 12000 g for 10 min and re-suspended in original liquid of 100μL. The divalent salts were pipetted to 96 well plates and detected by microplate reader (OD595). Another 5mL supernatants of each group were mixed with sodium acetate (2.5M, 600μL) and absolute alcohol (5mL) to precipitate DNA. The volumes were incubated on ice for 20min and followed by centrifugation at 12000 g for 10 min at 4°C. After washed with 75% alcohol (5mL) The precipitates were and dissolve in ddH2O (200μL). The DNA was detected by q-PCR and polyacrylamide gel electrophoresis (PAGE). The proteins in the volumes were precipitated by TCA Protein Precipitation Kit (Sangon Biotech, China) and detected by SDS-PAGE. The concentrations of phosphate in the mediums were measured by ammonium molybdate after 400 times diluted and calculated to its standard curve.

## Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All data generated during this study are included in this published article. The RNA sequencing results are available from the corresponding author for research.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: Hongsheng Ji and Zhikun Zhang designed the experiments and wrote the manuscript. Yingshun Zhou, Luhua Zhang and Ying Wang reviewed the manuscript. Feiyang Zhang, Jiawei Bai and Qin Li performed the experiments. All author contributed to data collection.

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# Figures

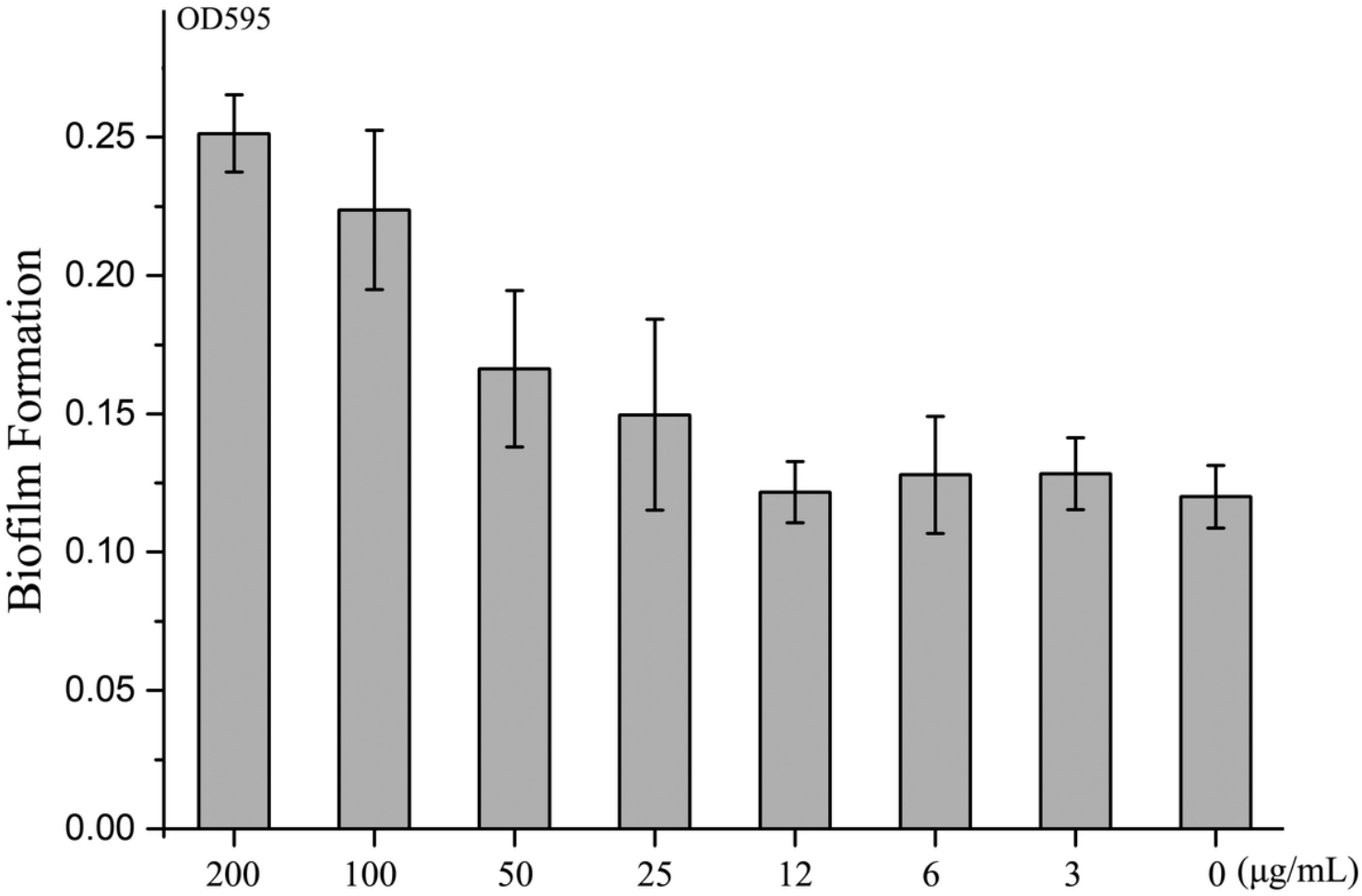
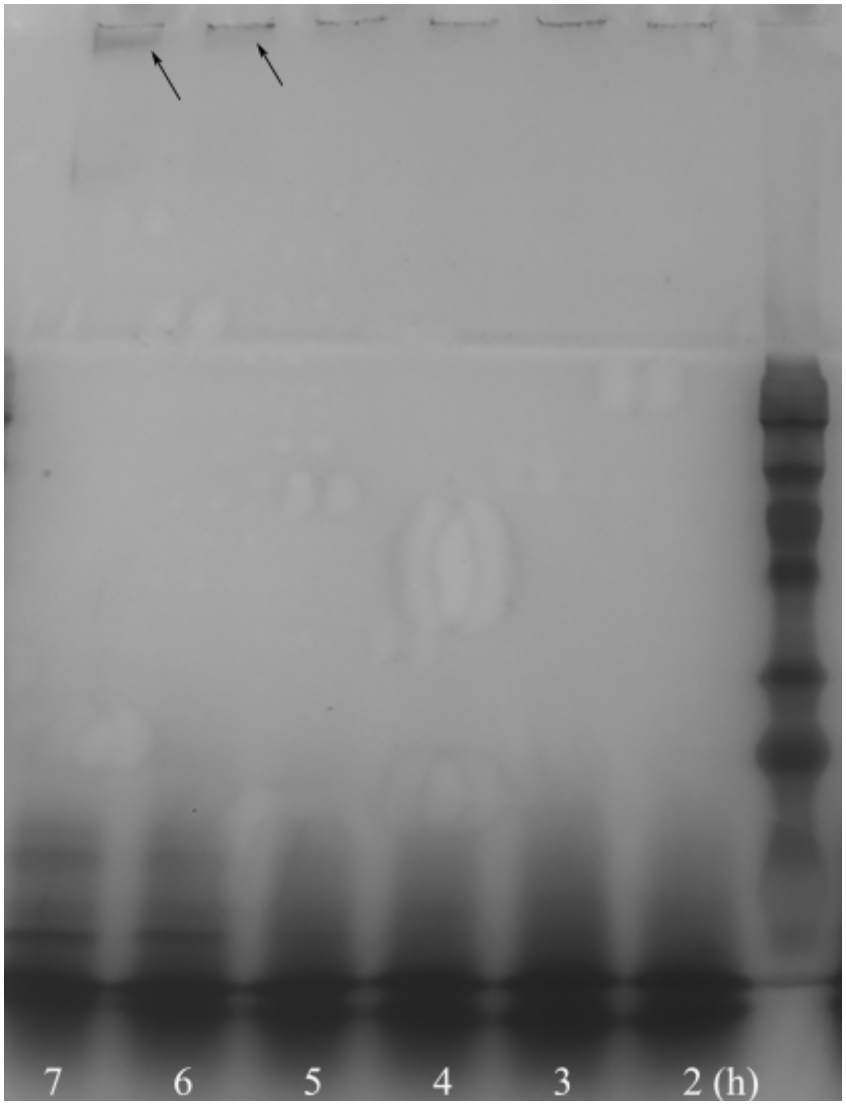


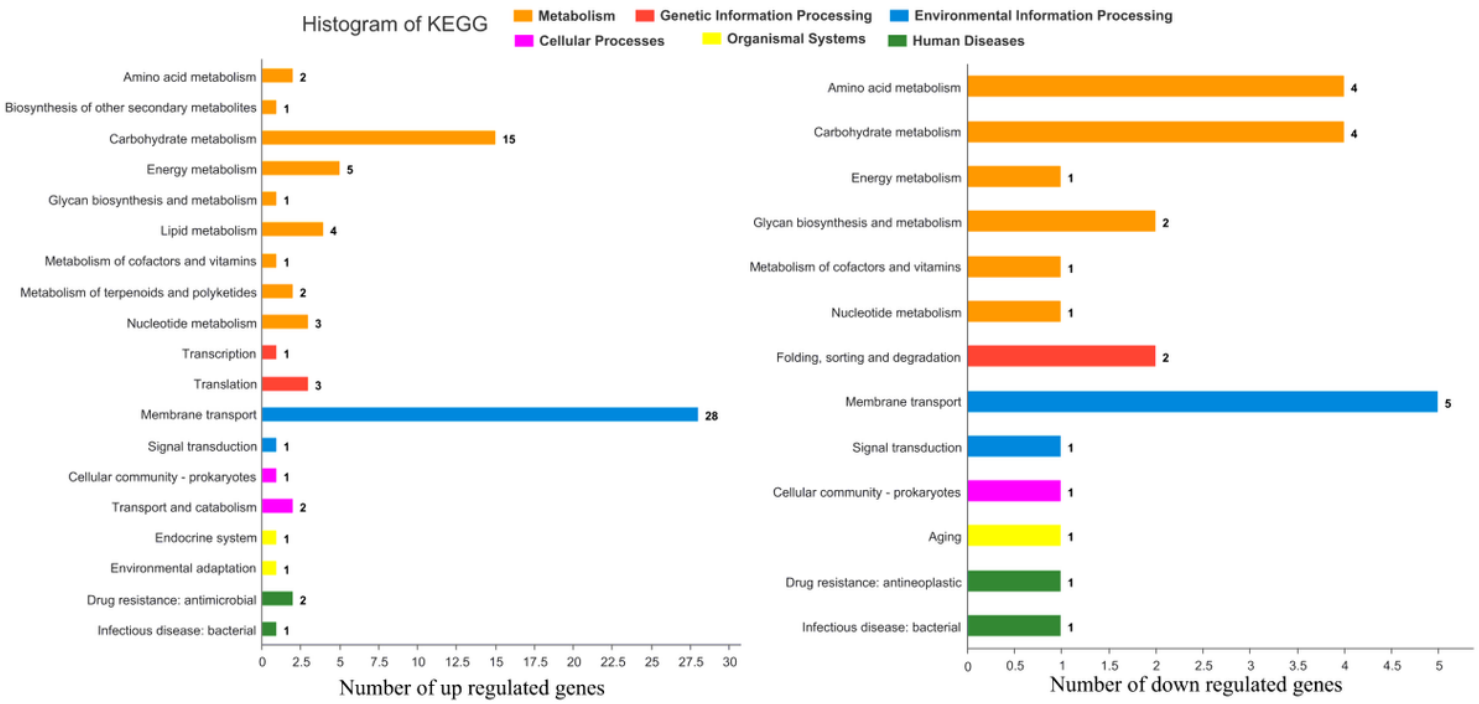

**Figure 1**

Effect of ChBp-I2 on biofilm formation of S. pneumoniae in vitro at different concentration.



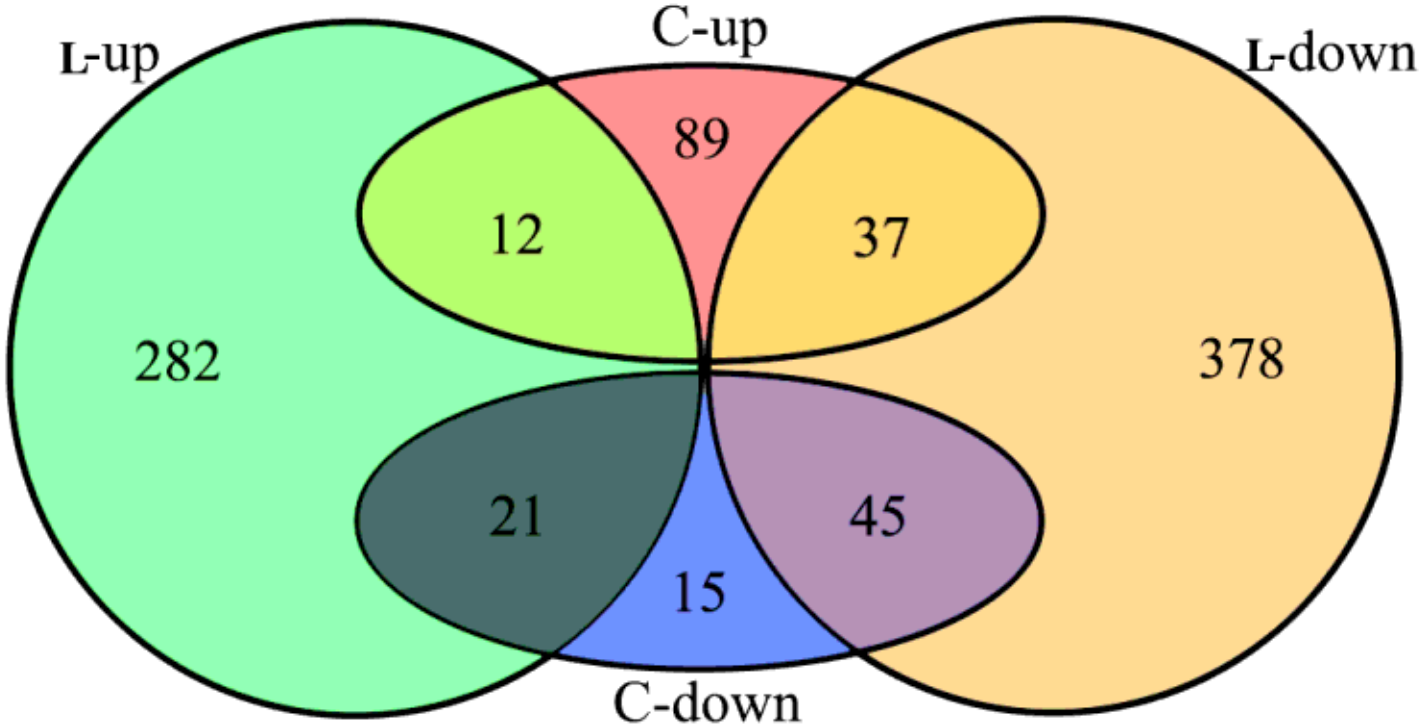

## Figure 2

Detection of extracellular choline binding proteins (CBPs) at different time point in vitro, the deduced CBPs were enriched by DEAE.



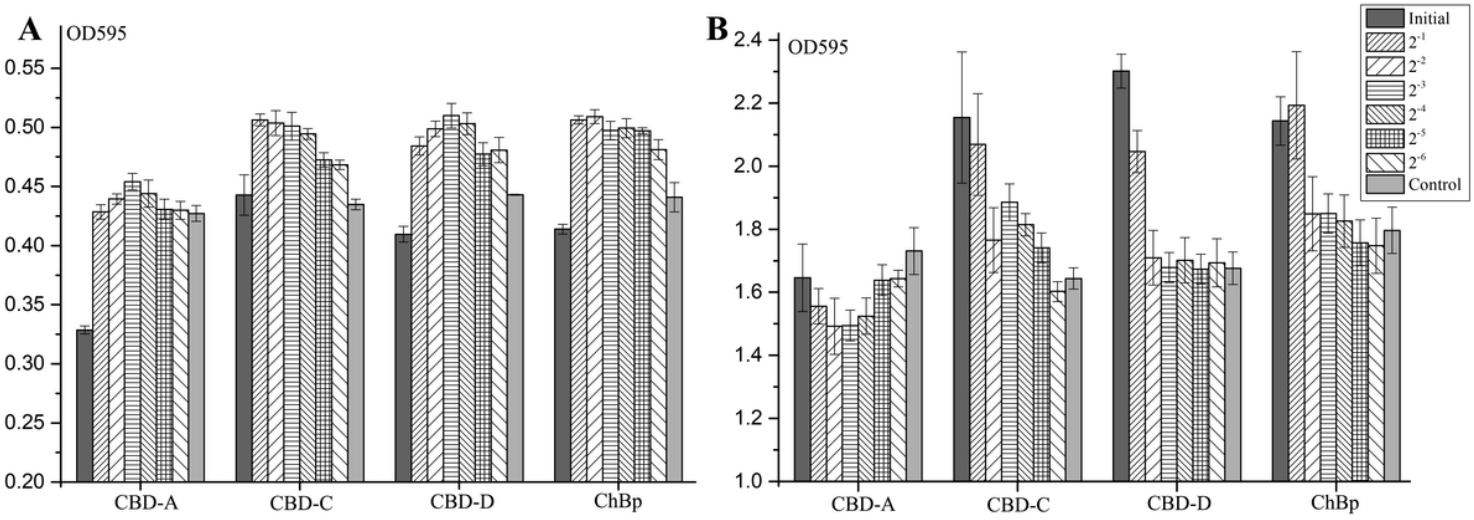

## Figure 3

KEEG analysis of the DE genes under ChBp-I2.



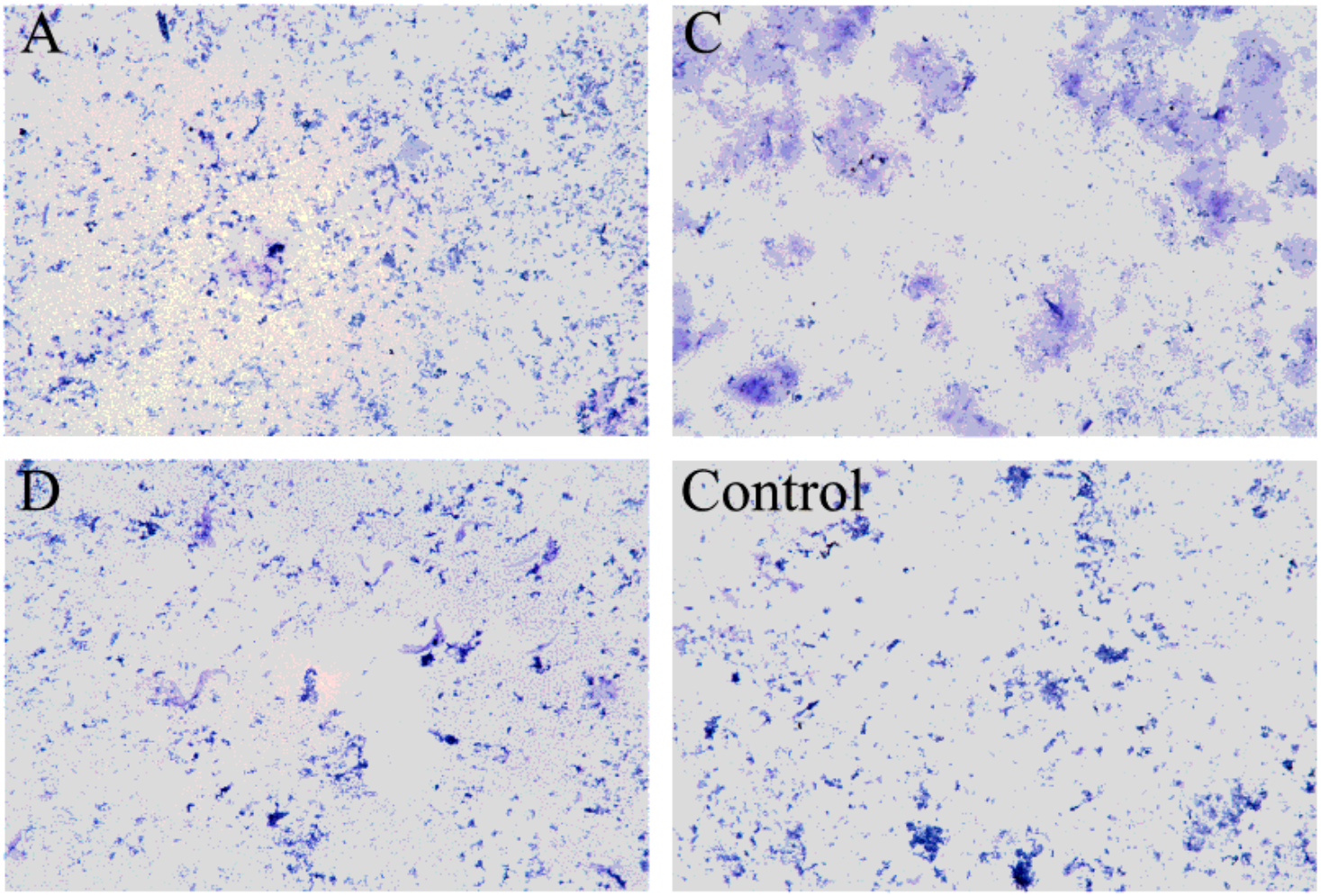

## Figure 4

Venn diagram of the DE genes under ChBp-I2 (C) and at late logarithmic phase (L).




## Figure 5

A: Growth and biofilm formation of pneumococcal cells under CBDs. (A): the OD values were tested at 2h post incubation CBDs. (B): Biofilm formations were detected at 6h post incubation. The initial concentrations of CBD-A, CBD-C, CBD-D are both 100μg/mL and ChBp is 25μg/mL.




## Figure 6

Biofilm formation by S. pneumoniae under the existence of CBD-A, CBD-C and CBD-D. Cells were incubated on tissue culture treated plated for 6h, and stained with CV.

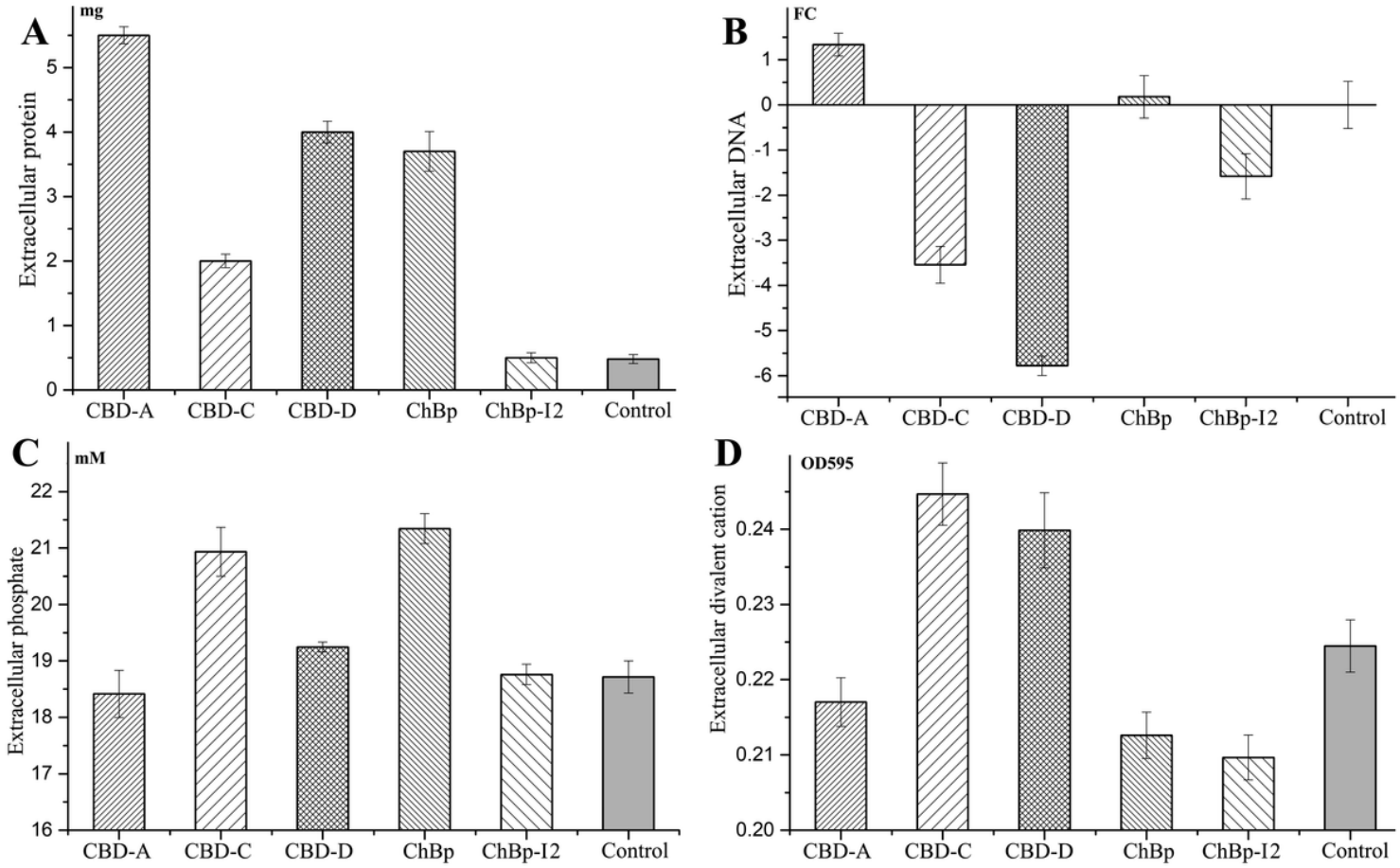



**Figure 7**

The content of extracellular protein (A), nucleic acid (B), phosphate (C) and divalent cation (D) after incubated in mediums containing CBD-A (100μg/mL), CBD-C (100μg/mL), CBD-D (100μg/mL), ChBp-I2 (50μg/mL) and ChBp (12.5μg/mL), using normal medium as control.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial.docx