IDENTIFICATION AND CHARACTERIZATION OF ANTIMICROBIAL AGENTS TO CONTROL BACTERIAL INFECTION

Flora Ianniello

Dottorato in Biotecnologiche – XXIX ciclo

Università di Napoli Federico II

Dottorato in Scienze Biotecnologiche – XXIX ciclo

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Dottoranda:	Flora Ianniello
Relatore:	Prof. Rosanna Capparelli
Coordinatore:	Prof. Giovanni Sannia

Alla mia famiglia
che mi ha sostenuto e che ancora
mi sostiene durante il mio percorso di vita…

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RIASSUNTO

Introduzione

Gli antibiotici sono farmaci di grandissimo rilievo che hanno migliorato sostanzialmente la vita di milioni di persone. Tuttavia, il loro uso eccessivo ed inappropriato ha provocato lo sviluppo di ceppi batterici antibiotico-resistenti, rendendo inefficienti i trattamenti terapeutici attualmente in uso (Livermore D.M. 2004). Secondo il rapporto dell'Ecdc (European Centre for Disease Prevention and Control), la resistenza agli antibiotici sia da parte dei batteri Gram-positivi che Gram-negativi è in rapida ascesa. Inoltre è stata riscontrata una notevole disparità tra l'aumento della frequenza di ceppi resistenti e la produzione di nuovi farmaci. Per far fronte a questa emergenza, è sempre più urgente sviluppare nuovi agenti terapeutici che abbiano una maggiore selettività ed un meccanismo d'azione diverso da quelli degli antibiotici attuali.

I peptidi antimicrobici e i batteriofagi potrebbero essere dei candidati ideali per la risoluzione del problema dell'antibiotico resistenza.

I peptidi antimicrobici (CAMPs) sono ampiamente distribuiti in natura, essi sono diversi in termini di lunghezza, di struttura primaria e secondaria, ma possiedono due caratteristiche comuni: a pH fisiologico sono cationici e anfipatici. La carica positiva rende i peptidi antimicrobici selettivi per la membrana batterica ricca di fosfolipidi carichi negativamente (fosfatidilserina, fosfatidilglicerolo e bisfosfatidilglicerolo) (Zasloff M. 2002). Inoltre, i peptidi antimicrobici, avendo una sequenza segnale simile a quella presente nelle proteine cellulari, non vengono degradate facilmente dalle proteasi (Boman HG. 2003). La membrana delle cellule eucariotiche a differenza della membrana batterica è ricca di fosfolipidi switterionici (fosfatidilcolina, sfingomielina), quindi l`interazione elettrostatica con i peptidi non può avvenire (Chia CS. et al., 2011). I CAMPs hanno un'azione antimicrobica ad ampio spettro: risultano attivi contro batteri Gram-positivi e Gram-negativi, virus e funghi (Lai Y. et al., 2009), costituiscono la prima linea di difesa (Boman H.G. 2000), mettono in relazione l'immunità innata con quella acquisita (Vizioli J. et al., 2002) ed inoltre possono agire sia come molecole antimicrobiche nei confronti dei patogeni, che come agenti immunomodulatori della risposta immunitaria (Scott MG. et al., 2007). Quest'ultima attività viene esplicata mediante: attivazione della chemiotassi, regolazione della produzione di citochine e stimolazione delle cellule del sistema immunitario, quali monociti e Antigen-Presenting Cells (APC). In aggiunta sono in grado di modulare il rilascio di istamina e migliorare l'angiogenesi, andando ad accelerare i meccanismi di cicatrizzazione delle ferite (Salzet M. et al., 2002; Chen Q. et al., 2004).

I batteriofagi o fagi sono parassiti intracellulari obbligati, in grado di moltiplicarsi solo all'interno della cellula batterica ospite. I batteriofagi furono scoperti da Hankins nel 1896, il quale in modo del tutto fortuito notò l'esistenza di questi "agenti antibatterici" nei fiumi Gange (Sulakvelidze A. et al., 2001) e Jumna. Nel 1917, Felix d'Herelle (ricercatore presso l'Istituto Pasteur di Parigi) dimostrò l'attività antibatterica dei batteriofagi sulla *Shigella dysenteriae* nei conigli e chiamò questi agenti antimicrobici batteriofagi ("*mangiatori*" di batteri). Nel 1930, d`Herelle fondò in Georgia un istituto per lo studio dei batteriofagi, dove ancora oggi, i fagi sono regolarmente utilizzati per controllare le infezioni batteriche nell'uomo (Clokie M. et al., 2011). Questi procarioti sono la forma di vita più abbondante nella biosfera (Brüssow H. et al., 2005), ogni centimetro cubo di acqua contiene da 10 a 100 milioni di fagi (Sulakvelidze A. et al.,

2001). Questi ultimi sono molto diversificati e questa diversità è determinata dalla loro capacità di adattamento dinamico per superare la pressione selettiva. Infatti, quando i fagi infettano le cellule batteriche, si trovano ad affrontare i meccanismi di difesa antivirali, per questo hanno sviluppato varie strategie per evitare, eludere o sovvertire questi meccanismi. I fagi possono essere classificati in base alla morfologia, al genoma, ed alla gamma degli ospiti. Il materiale genetico può essere dsDNA, ssDNA o RNA. La maggior parte dei fagi hanno un capside icosaedrico (contenente DNA) legato attraverso il collare ad una coda che può avere le spine e/o fibre; avendo un meccanismo di azione più specifico rispetto agli antibiotici, i fagi non comportano danni per l'organismo umano e per i batteri benefici, come quelli della flora intestinale (Merril CR. et al., 2003). Inoltre, essendo dei parassiti obbligati hanno il vantaggio di presentare una crescita esponenziale che è direttamente proporzionale alla carica microbica presente durante l'infezione (Merril CR et al., 1996), per contro hanno come svantaggio quello di avere un' emivita breve e di essere instabili senza il loro ospite. Per ovviare a questo loro limite, nasce l'esigenza di realizzare un sistema (scaffold) che sia in grado di stabilizzare il fago, aumentarne l'emivita e veicolarlo all'interno delle cellule eucariotiche.
Una molecola ideale, con le suddette caratteristiche, potrebbe essere l'Idrossiapatite Ca_5 $(PO_4)_3$ (OH): raro minerale di forma esagonale appartenente al gruppo dell'apatite. Questi nanocristalli presentano eccellenti proprietà biologiche (Roveri N. et al., 2006; Barroug A. et al., 2003), non sono tossici, non inducono la risposta infiammatoria (Iafisco M. et al., 2008) ed hanno un'elevata attitudine a stabilizzare/veicolare molecole e farmaci bioattivi (Akawa T. et al., 1999).

Scopo della tesi

La scoperta degli antibiotici nel 1928 ha salvato milioni di vite e alleviato la sofferenza di molti pazienti. Ora, quasi 90 anni dopo, siamo nell'era "post-antibiotica", in cui diverse infezioni batteriche non possono essere trattate con successo perché anche i farmaci più potenti sono ormai inutili. Epidemie infettive causate da batteri resistenti sono numerosissime, la più conosciuta è la pandemia globale di infezione da *Staphylococcus aureus* resistente alla meticillina (MRSA) (Moran GJ. et al., 2006). Il sistema sanitario degli Stati Uniti investe circa $ 34 miliardi ogni anno per sradicare questo patogeno (Filice GA. et al., 2010), più letale di malattie come l' HIV/AIDS, morbo di Parkinson, e l'enfisema (Klevens RM. et al., 2007). Negli Stati Uniti ed in altre parti del mondo è comune l'utilizzo di antibiotici in agricoltura e negli allevamenti come promotore di crescita (Levy SB. et al., 2002). Questo uso inappropriato contribuisce all'aumento della resistenza agli antibiotici sia per i Gram-negativi che per i Gram-positivi. La produzione di antibiotici non è di rilevante interesse economico per le aziende farmaceutiche in quanto, i batteri hanno la capacità di sviluppare resistenza agli antibiotici velocemente, rendendo il loro uso sempre meno funzionale. In questo studio si propone la possibilità di utilizzare batteriofagi o peptidi antimicrobici per contrastare le infezioni batteriche, in campo farmaceutico come nuova terapia e nel campo agroalimentare per ridurre l'uso di conservanti sintetici e contemporaneamente aumentare la shelf-life dei prodotti.

Disegno sperimentale
Lo studio è stato articolato in quattro fasi:

1. Identificazione, produzione e valutazione dell'attività antimicrobica di PAP3

Negli ultimi anni, sono state scoperte diverse proteine che mostrano attività antibatterica non legata alla loro funzione primaria. Queste proteine sembrano agire come vettore di peptidi antimicrobici criptici, che potrebbero essere rilasciati dalle proteasi umane o batteriche. I peptidi antimicrobici criptici sono spesso situati all'estremità N-o C-terminale della proteina, quindi durante il processo di maturazione possono essere liberati. Per identificare rapidamente peptidi antimicrobici criptici, il Dr. Notomista E. (Dipartimento di Biologia, Università di Napoli Federico II), ha sviluppato un sistema di previsione che correla le caratteristiche strutturali dei CAMPs con le loro proprietà antimicrobiche. Il sistema di previsione è stato utilizzato per analizzare le proteine secrete umane depositate negli appositi database. Con questa analisi sono state identificate come potenziali peptidi antimicrobici le porzioni N-terminale dell'isoforme A3, A4 e A5 del pepsinogeno umano. Il pepsinogeno è costituito da una singola catena polipeptidica di peso molecolare di circa 42 000 Da contenente tre legami disolfuro ed un punto isoelettrico di 3.7. Esso è costituito da tre regioni: un peptide segnale (circa 15-16 aa), un segmento di attivazione (circa 47 aa) e la parte attiva dell'enzima (326 aa). Dall'allineamento delle sequenze amminoacidiche è emerso che le tre isoforme differiscono per un amminoacido: l'isoforma A3 ha un residuo di lisina (K43), mentre le isoforme A4 e A5 hanno un residuo di acido aspartico (E43). Pertanto, il pro-peptide dell' isoforma A3 del pepsinogeno (PAP3) ha due cariche positive in più rispetto alle isoforme A4 e A5. Questa caratteristica rende l'isoforma A3 particolarmente interessante per questo studio. Per la produzione di PAP3 è stato utilizzato un sistema di espressione procariotico (*E. coli*) che rappresenta una valida alternativa alla sintesi chimica. Per evitare che il peptide antimicrobico potesse risultare nocivo per le cellule di *E. coli,* durante la fase di sintesi (Li Y. et al., 2009), abbiamo scelto di esprimere il peptide PAP3 come proteina di fusione posizionandolo al C-terminale dell'onconasi (ribonucleasi) di rana. Per separare il peptide dall'onconasi è stato necessario interporre fra le due parti una sequenza **GTGDP** linker, che come è noto in letteratura, a 60-80℃ in ambiente acido viene deg radata, generando frammenti che contengono al N –Terminale un residuo di Prolina. Inoltre la proteina di fusione, presentando una sequenza di 6 residui di istidina situati all'estremità C-terminale dell' onconasi e l'N-terminale del peptide, ha permesso la purificazione della proteina mediante cromatografia di affinità per chelazione di metalli (IMAC).
L'onconasi è un partner adatto per diversi motivi: i livelli di produzione risultano essere molto elevati come corpi di inclusione (circa 150 mg/L in brodo); nessun onconasi solubile può essere rilevata nelle culture (minimizzando così il rischio di effetti tossici del peptide); ed è una piccola proteina (104 aa) permettendo così rese di produzione di PAP3 più elevate. Inoltre l'onconasi estratta dai corpi di inclusione è solubile a pH acidi (<4) ma completamente insolubile a pH 7. Quindi il clivaggio della proteina di fusione è stato effettuato a pH acido e successivamente il pH è stato innalzato fino a 7 utilizzando l'ammoniaca (NH_3). In questo modo l'onconasi precipita mentre PAP3 rimane in soluzione. La proteina ricombinante è stata espressa in *E. coli* ceppo BL21DE3 attraverso 3 ore di induzione con IPTG ad una concentrazione finale di 0,4 mM; alla fine di questa fase, le cellule sono state sottoposte a lisi

meccanica mediante ultrasuoni e la porzione solubile (contenente proteine di *E.coli*) è stata rimossa mediante centrifugazione. Inoltre i componenti delle membrane cellulari sono stati eliminati effettuando un trattamento con detergenti e denaturanti come Triton X-100 e urea. Al termine di questi trattamenti la frazione contenente corpi di inclusione insolubili è stata sottoposta a denaturazione e riduzione con guanidina-HCl 6 M e beta-mercaptoetanolo 10 mM. Il campione è stato successivamente centrifugato e la frazione solubile è stata purificata mediante cromatografia di affinità IMAC. Per distaccare l'onconasi da PAP3 è stata effettuata un idrolisi acida eseguita a pH 2 a 60℃ e successi vamente il pH è stato innalzato fino a 7 per allontanare l'onconasi (solubile solo in ambiente acido). Le frazioni (solubile e insolubile) sono state analizzate mediante SDS-PAGE al 20%. È stata riscontrata la presenza dell'onconasi solo nella frazione insolubile, mentre il peptide era presente solo in quella solubile. Il peptide PAP3 purificato è stato analizzato mediante cromatografia in fase inversa su colonna C18 e dal cromatogramma è stato possibile apprezzare la presenza di un picco principale eluito a 52.28 min, risultato essere PAP3 attraverso elettroforesi su SDS-PAGE al 20%.
L'attività antimicrobica di PAP3 è stata saggiata su alcuni dei principali patogeni alimentari *Listeria monocytogenes, Salmonella rissen, Salmonella typhimurium*, *Staphylococcus aureus, Escherichia coli.* Dai risultati ottenuti è stato possibile osservare che PAP3 alla concentrazione di 220 µg/mL inibisce la crescita dei batteri in un range del 90-100%. Questi dati forniscono la prova che PAP3 (come già previsto da strumenti bioinformatici) è un buon peptide antimicrobico, efficace sia su batteri Gram-negativi che Gram-positivi.

2. Isolamento, caratterizzazione e stabilizzazione dei batteriofagi

Sono stati isolati batteriofagi dai principali patogeni alimentari: *Salmonella rissen, Salmonella monofasica, Listeria monocytogenes, Escherichia coli* e *Staphylococcus aureus.* I batteriofagi isolati sono stati caratterizzati tramite host range (range di attività litica), curva di crescita e MOI (Minima concentrazione di fago necessaria per l' infezione del batterio). Il fago della *Salmonella rissen* ci ha fornito risultati migliori: host range 11/14 ceppi analizzati, fase di latenza e quella di crescita rispettivamente di 30 e 55 min, il burst size di 54 particelle fagiche e anche ad una MOI bassa come 0,001 è stata riscontrata una buona attività litica. Il fago della *Salmonella rissen* Φ1 data la miglior risposta ai test è stato scelto per eseguire esperimenti successivi. Attraverso l'utilizzo della microscopia elettronica è stato possibile affermare che il batteriofago Φ1 fa parte della famiglia *Podoviridae*. Esso ha una testa di diametro 57 ± 1, 0 nm, una coda flessibile di 16 ± 2, 0 nm e la sua lunghezza totale è di 73 ± 2, 0 nm. Il fago Φ1 ha dsDNA di 42,990 bp, costituito da 65 sequenze codoniche (CDSS) e contenente il 48,6% di G+C.
Come precedentemente descritto i fagi essendo parassiti obbligati, non sono stabili ed hanno un'emivita breve senza il loro ospite. Per questo motivo abbiamo provato a stabilizzare il fago Φ1 con l'ausilio dell'idrossiapatite (HA).

I nanocristalli di idrossiapatite biomimetica (HA) [Ca_5 $(PO_4)_3$ (OH)] sono stati precipitati da una soluzione acquosa di $(CH_3COO)_2Ca$ mediante lenta aggiunta di H_3PO_4, mantenendo costante il pH a 10 per aggiunta di (NH_4) OH (Palazzo B. et al., 2009). Per produrre HA con il rapporto stechiometrico Ca/P di 1.67, la sospensione di Ca $(CH_3COO)_2$ e la soluzione di H_3PO_4 utilizzati erano rispettivamente 83 e 50

mM. La miscela di reazione è stata agitata a 37℃ e dopo 24h è stata lasciata in condizioni statiche per 2 ore per permettere la deposizione della fase inorganica. Quest'ultima è stata lavata ripetutamente con acqua e liofilizzata a -60℃ sotto vuoto (3 mbar) per 12 ore. L'idrossiapatite biomimetica prodotta è risultata simile a quella riscontrata nel corpo umano. Essa rivela una morfologia piastriforme, con lunghezza, larghezza e spessore di circa 110 ± 5 nm, 20 ± 3 nm e 8 ± 2 nm. L'elevata reattività dell' idrossiapatite è attribuita alla sua superficie amorfa, ed alla sua elevata area superficiale di circa 110 m^2/g, che è solo di poco inferiore a quella dei nanocristalli biologici (120 m^2/g). Quando i nanocristalli di HA sono a pH 7,4 mostrato un potenziale zeta di 20,5 ± 1,5 mV, questo valore è attribuito alla loro superficie amorfa. Sono state valutate diverse condizioni per la formazione del complesso fago-idrossiapatite (Φ-HA), quella migliore ha previsto l'utilizzo 1ml di HA (100 mg/mL) e 1 ml di Φ (10^8 PFU/mL). Dopo l'incubazione overnight il complesso è stato centrifugato a 5000 rpm per 5 minuti, il surnatante (contenente la porzione di fago che non si è legato all'HA) è stato eliminato mentre invece il pellet è stato risospeso in 1ml di buffer SM (20 mM Tris-HCl [pH 7.2], 10 mM NaCl, 20 mM $MgSO_4$).
Gli studi condotti mediante spot test, hanno evidenziato una maggiore stabilità del complesso fago-idrossiapatite rispetto al solo fago infatti dopo diversi mesi conservato a + 4℃, il fago complessato con l'idros siapatite mantiene costante il suo titolo espresso in PFU/mL. Inoltre il complesso resta attivo anche in ambiente acido, per questa ragione, potrebbe essere idoneo per combattere le infezioni nel tratto gastro-intestinale.

3. Test per valutare la citotossicità

Sulle cellule del carcinoma del fegato (HepG2) sono stati effettuati due test distinti (MTT e LDH) per valutare la citotossicità del peptide PAP3 (220 µg/mL), del fago Φ1 (10^8 PFU/mL), del complesso (10^8 PFU/mL) e dell'idrossiapatite (100 mg/mL). Il test MTT (bromuro di 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio) ha dimostrato che PAP3 è tossico per le cellule eucariotiche, mentre gli altri trattamenti non influenzano la vitalità cellulare: le cellule sono risultate vitali anche dopo 72 ore dal trattamento. LDH test ha evidenziato una bassa produzione dell'enzima lattato-deidrogenasi per ciascuna condizione sperimentale fino a 72 ore, tranne per PAP3, il quale ha determinato un significativo incremento di LDH. Con questi risultati è possibile affermare che il fago Φ1, il complesso e l'idrossiapatite non comportano nessuno stress alle cellule eucariotiche del carcinoma del fegato, mentre PAP3 è risultato citotossico.

4. Attività antimicrobica nelle cellule eucaristiche e nella carne

Per valutare l'attività intracellulare sono stati effettuati due esperimenti distinti. In uno le cellule sono state infettate con *Salmonella rissen* (10^4 CFU/mL), trattate con gentamicina (12,5 mg/mL) e solo dopo 3 h di incubazione a 37℃, sono stati effettuati i trattamenti con il fago Φ1 (10^8 PFU/mL), il complesso (10^8 PFU/mL) e la sola idrossiapatite (100 mg/mL). Nel secondo esperimento, l'infezione con la Salmonella e i diversi trattamenti sono stati effettuati contemporaneamente. Dopo 18h di incubazione a 37℃, le cellule di entrambi g li esperimenti sono state lisate ed il lisato è stato spottato su piaste XLT4. I risultati del primo esperimento evidenziano una efficace attività antimicrobica solo per il complesso (Φ-HA), mentre il fago e l'idrossiapatite da soli non hanno determinato nessuna riduzione della carica

microbica. Dai risultati ottenuti dal secondo esperimento (dove è stata effettuata l'infezione contemporaneamente ai trattamenti), è stato possibile valutare che anche il fago come il complesso (Φ-HA), ha determinato una sostanziale diminuzione della crescita batterica. Con questi risultati è possibile sostenere che il fago da solo non ha la capacità di entrare nelle cellule eucariotiche e solo quando è veicolato dall'idrossiapatite o dal batterio (nel nostro caso la *Salmonella rissen)* riesce ad uccidere i batteri intracellulari. Invece il complesso (Φ-HA), è capace di entrare all'interno delle cellule eucariotiche grazie all'azione dell'idrossiapatite, che tramite un meccanismo di endocitosi determina l'ingresso del fago nel citoplasma delle cellule eucariotiche (Bauer I.W. et al., 2008).

Per saggiare l'attività antimicrobica negli alimenti, del fago Φ1, del complesso (Φ-HA), e dell'idrossiapatite, un campione di carne da 250 gr è stato suddiviso in 5 porzioni uguali, di cui 4 sono state infettate con 20 ml di *Salmonella rissen* (10^3 CFU/mL) ed incubate a temperatura ambiente per un corretto fissaggio batterico. La quinta porzioni è stata trattata solo con 5 ml di PBS (controllo negativo). Dopo 30 minuti di incubazione 3 delle 4 porzioni infettate sono state trattate rispettivamente con il fago Φ1 (10^8 PFU/mL), il complesso (10^8 PFU/mL) e l'idrossiapatite (100 mg/mL), mentre la quarta non ha subito nessun trattamento (controllo positivo). Tutti i campioni sono stati conservati a 4℃ per 7 giorni e analizzati ad intervalli regolari di 24h. Ad ogni intervallo di tempo, 5 gr di carne (prelevati da ogni da ogni campione) sono stati omogeneizzati in 45 ml di acqua peptonata (PW) allo 0,1% utilizzando lo Stomacher 400 Circulator, Seward Ltd. Successivamente 100 µl di campione sono stati piastrati su XLT4 agar ed incubati a 37℃ per 24h. L'efficacia dei singoli trattamenti è stata valutata mediante conta delle CFU/gr. Le colonie di Salmonella nel campione trattato con il fago sono state ridotte di 0.3 log CFU/gr mentre il campione trattato con l'idrossiapatite aveva la stessa carica di Salmonella del controllo positivo 5.5 log CFU/gr. Grazie al complesso (Φ-HA), invece, la carica microbica nella carne è stata ridotta di 3 log CFU/gr.

Conclusioni

L' antibiotico resistenza è uno dei maggiori problemi di interesse socio economico che coinvolge il mondo scientifico e il mondo farmaceutico. Questo problema, riflette l'uso inadeguato di questi farmaci "miracolosi" e la mancanza di nuove terapie antimicrobiche in grado di tenere il passo con l'evoluzione dei batteri. Interventi legislativi per modulare l'uso di antibiotici sono all'orizzonte, ma per risolvere veramente questa crisi farmacologica sono necessari nuovi approcci scientifici. In questo lavoro di dottorato sono state proposte due alternative agli antibiotici: i peptidi antimicrobici e i batteriofagi.

Il peptide antimicrobico utilizzato (PAP3) è stato identificato dal sistema di previsione, aveva una buona attività litica in vitro contro vari agenti patogeni, ma è risultato tossico per le cellule eucariotiche anche a bassa concentrazione. Per questo motivo non è stato utilizzato per ulteriori esperimenti.

I batteriofagi sono stati isolati con successo da diversi agenti patogeni alimentari come *Salmonella typhimurium*, *Salmonella rissen*, *Listeria monocytogenes*, ed *Escherichia coli*. Il fago Φ1 (isolato da *Salmonella rissen*), è stato scelto per ulteriori studi in vista del suo ampio host range e della sua buona attività litica. Utilizzando la microscopia elettronica e analizzando la sequenza di DNA, il fago Φ1 è stato classificato nella famiglia *Podoviridae* e il suo genoma è risultato diviso in tre parti (preearly, early, and late regions) come il fago T4.

Il problema è che i fagi hanno una breve emivita e non possono essere utilizzati contro infezioni intracellulari, poiché non sono in grado di penetrare la membrana delle cellule eucariotiche. Inoltre, i fagi non sopravvivono in ambienti a pH acidi come quello dello stomaco e la loro vitalità può essere compromessa quando impiegati durante le fasi di lavorazione e di conservazione degli alimenti (Colom J. et al., 2017). Questi problemi sembrano risolvibili complessando i fagi con l'idrossiapatite: il complesso risulta essere stabile e capace di attraversare la membrana delle cellule eucariotiche. Inoltre, il Φ-HA è in grado di sopravvivere per più di 24 ore a pH estremamente acidi e di controllare l'infezione da Salmonella nella carne. Sulla base dei risultati ottenuti, si ritiene che il complesso (Φ-HA) possa essere considerato uno strumento biotecnologico efficace per il trattamento e/o la prevenzione delle infezioni batteriche.

Studi futuri riguarderanno l'analisi chimica-fisica per caratterizzare l'interazione tra il fago e l'idrossiapatite e un eventuale utilizzo dello stesso complesso associato ai peptidi antimicrobici

SUMMARY

The inappropriate and excessive use of antibiotics in medicine and agriculture led to the development of antibiotic resistant bacteria as shown by the data presented by the ECDC (European Centre for Prevention and Disease Control) and the HI (Health Institute). The development of new therapeutic agents is necessary to cope with this emergency. The characteristics demanded to new antibacterial drugs are greater selectivity and a different mechanism of action compared to traditional drugs in use.

The peculiar properties of peptides and phages make their use attractive to counter the problem of antibiotic resistance. The CAMPs are widely distributed in nature and differ in terms of length, primary and secondary structures, but they all have the characteristics of being cationic and amphipathic. The CAMPs have as " target" the bacterial cell membrane. This structure is highly conserved among bacteria and its reorganization would be highly expensive and difficult to realize.

Bacteriophages are viruses that specifically infect bacterial cells. They do not harm the natural flora of the human host when used as antimicrobial agents. In this study, we used the antimicrobial peptide PAP3 (A3 isoform of human pepsinogen) and the phage Φ1 (isolated by *Salmonella rissen*).

PAP3 displays a strong antimicrobial activity against several bacterial species (at the concentration of 220 µg/mL inhibits 90%-100% of bacteria). Unfortunately, PAP3 is toxic for eukaryotic cells, even at low concentration and for this reason it has not been studied further. On the contrary, the phage Φ1, - isolated and characterized in the course of this study - is a good antimicrobial agent. To the same extent of all the other phages, Φ1 is species-specific, is not toxic to eukaryotic cells and one single dose of phage is sufficient to control bacterial infection. To date, no phage preparation for intracellular pathogens is available. In this study – for the first time – it was tested the use of hydroxyapatite as a vehicle to carry Φ1 inside the eukaryotic cell. It was shown that hydroxyapatite carries the phage inside eukaryotic cells and does it without damaging the cells. The hydroxyapatite, $Ca_5 (PO_4)_3 (OH)$, is a rare mineral of the apatite group. It has the shape of a hexagonal prism and serves as protective scaffold and as carrier of bioactive molecules. Our results show that the complex (phage-hydroxyapatite) is more stable than phage alone, is active at pH 2 and is a good agent to reduce bacterial contamination in the food.

Based on our results, we believe that the use of bacteriophages complexed with hydroxyapatite represents a promising biotechnological approach to treat /to prevent bacterial infections in several areas.

1. INTRODUCTION

1.1 Antibiotic

Antibiotics are natural or synthetic substances, which can control bacterial infections (bactericide antibiotics) or inhibit their growth (bacteriostatic antibiotics). Based on their spectrum of action, antibiotics can have a broad spectrum (when are active against Gram positive and Gram negative bacteria) or narrow-spectrum (in the case of molecules active against a restricted number of bacterial species). Before the discovery of this important class of drugs, many "harmless" disease led to death (seasonal virus, pneumonia, septicaemia, tuberculosis). The birth of antibiotics dates back to many centuries ago. Greeks and Egyptians took care of febrile infections with natural substances, and in 1700 moulds began to be used. In 1868 Pasteur began the "fight" against bacteria. In 1900 Paul Ehrlich experimented a compound effective against the syphilis bacterium. In this research climate in 1928, the English microbiologist Alexander Fleming noted that a mould contamination destroyed a bacterial culture completely. Fleming identified the mould as belonging to the *Penicillium notatum* species. In the 40s this wonder drug was spread all over the world and Fleming won the Nobel Prize for medicine in 1945 (Blaser MJ. 2016). In the following years different chemical researchers discovered other molecules with antibacterial activity and their efforts greatly improved the quality of life of humankind. Inappropriate and excessive use of antibiotic led to the development of resistant strains (Pamer EG. 2016). In 1952 a multidrug-resistant Shigella strain (tetracycline-, streptomycin- and sulphonamides-resistant) was isolated. In 1959 the multiple resistance to these antibiotics was transferred to *E. coli*. Today antibiotic resistance an increasingly important medical problem. The antibiotic-resistance assumes great importance also in the food industry. The EFSA (European Authority for Food Safety) has reported that resistance to nalidixic acid in *Salmonella enterica* has grown from 13% to 15%. Microorganisms have several alternatives to avoid the lethal action of antibiotics (Sibhghatulla S. et al., 2015) (Fig.1) through:

a) production of enzymes able to inactivate antibiotics (many pathogens produce β-lactamases, this enzyme hydrolyses the lactam-ring β, destroying the antibacterial activity of antibiotics);

b) alteration of the permeability of the casing (in this way the drug cannot penetrate the bacterial cell);

c) modification of the target (antibiotic target is modified by replacing single amino acid, acquisition of a gene and methylation);

d) active transport systems (through this system the antibiotic is transported outside of the bacterial cell);

e) alternative metabolic pathways.

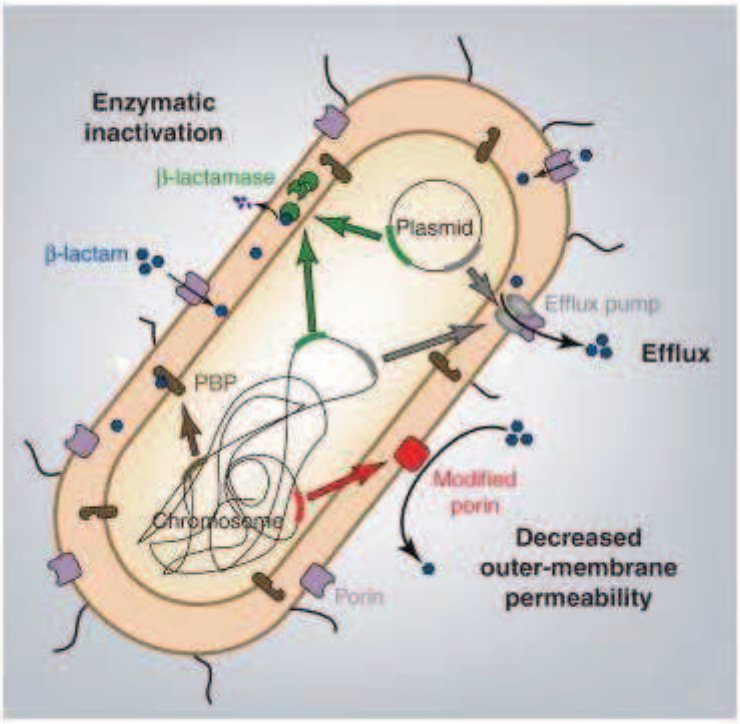

Figure 1: Resistance mechanisms of bacteria (http://www.cell.com/cms/attachment/2002995576/2011441548/gr1.jpg).

The development of new therapeutic agents is necessary to cope with these emergencies. The characteristics demanded to the new antibacterial drugs are greater selectivity and a different mechanism of action compared to traditional drugs in use. The antimicrobial peptides and bacteriophages could be the answer to this emergency.

1.2 Antimicrobial peptides

The cationic antimicrobial peptides (CAMPs) are small molecules widely distributed in nature, synthesized by organisms belonging to the plant and animal kingdoms. The CAMPs derive from a pro-peptide of approximately 60-170 amino acids and are then released into the mature form after the action of specific proteases that produce amino acid sequences of a variable number of residues (10-50) (Gautier M.F. et al., 1994). They are different in terms of length, primary and secondary structure, but are both cationic and amphipathic. These features allow the peptides to interact with the cell membrane, forming ion channels or pores or dissolving the membrane, like detergents (Rinaldi A. C. et al., 2002). The antimicrobial peptides constitute the first defence line of the organisms against a large variety of external agents (Boman H.G. 2000) and in higher organisms link the mechanisms of innate and acquired immunity (Vizioli J. et al., 2002). The CAMPs directly destroy pathogens (Scott MG. et al., 2007), but they modulate also the response of the immune system, activating chemotaxis, regulating cytokine production and stimulating the immune system cells, such as monocytes and antigen-presenting cell (APC). Furthermore, they are also able to modulate the release of histamine and enhance angiogenesis, accelerating

the healing of wounds (Salzet M. et al., 2002; Chen Q. et al., 2004). The innate immunity peptides rapidly respond against infectious agents (Tossi A. et al., 2002). In mammals, they act earlier than the antibody response or cell-mediated immunity, CAMPs are able to kill bacteria within 20 minutes from treatment (Piers KL. et al., 1994). Insects under normal conditions show a low level of antimicrobial peptides, but upon exposure to pathogens peptides production rapidly increases (Hoffmann JA et al., 1996; Levy F. et al., 2004).
Some antimicrobial peptides exert different functions. For example, the puroindoline (antimicrobial proteins of wheat belonging to defensins family) act against different types of pests and mushrooms (Dubreil L. et al., 2002; Capparelli R. et al., 2005; Capparelli R. et al., 2006), and they also control the wheat grain hardness (Giroux MJ. et al., 1998). CAMPs act on Gram-positive, Gram-negative bacteria, and also on viruses, fungi (Lai Y. et al., 2009) and cancer cells (Tomasinsig L. et al., 2006; Hoskin DW. et al., 2008). The action of antimicrobial peptides depends on the amino acid sequence (Zhao H. et al., 2002), and from lipids that make up the bacterial membrane (Brian M. et al., 2010). The net charge of antimicrobial peptides is directly correlated with their attraction / interaction with the bacterial membrane. In fact, a cationic antimicrobial peptide, which has a net positive charge at physiological pH (Shai Y. et al., 1996; Oren Z. et al., 1998; Maher S. et al., 2006), can easily interact with the negatively charged bacterial surface (Brogden K.A. et al., 2007). In contrast, the interactions of cationic peptides with mammalian cell membranes are significantly weaker because they are rich in phospholipids zwitterionic (free of charge), such as phosphatidylethanolamine, phosphatidylcholine and sphingomyelin (Yeaman M.R. et al., 2003). Moreover, the presence of cholesterol in the cell membrane of mammals, reduces activity of the peptides (Matsuzaki K. et al., 1996). The bacteria hardly develop resistance to the antimicrobial peptides (Thevissen K. et al., 2000; Boman HG. et al., 2003; Levy F. et al., 2004) because the CAMPs act against the bacterial membranes with different mechanisms respect the common drugs. Three important interaction models were developed: the model "barrel-stave", "carpet" and "toroidal pore" (Fig.2).

***Carpet model*:** In this model, the peptide is positioned parallel to the double layer of membrane phospholipid (Bechinger B. et al., 1999), with the hydrophobic groups in contact with the heads of anionic phospholipids of the bacterial membrane and the hydrophilic facing outward. These interactions determine the rupture of the bacterial membrane (Oren Z. et al., 1998; Shai Y. et al., 2002).

***Barrel stave model*:** The peptides are arranged perpendicularly to the bacterial membranes, with the hydrophobic groups toward the anionic heads of the phospholipids. In this case the peptide kills the microorganisms by puncturing the bacterial membrane.

Toroidal pore model: it combines the actions of barrel-stave and carpet model (Yang L. et al., 2001). The peptides are arranged on the bacterial membrane as occurs in the carpet model, after they are inserted perpendicularly into bacterial membrane causing deformation (Matsuzaki K. et al., 1996*).*

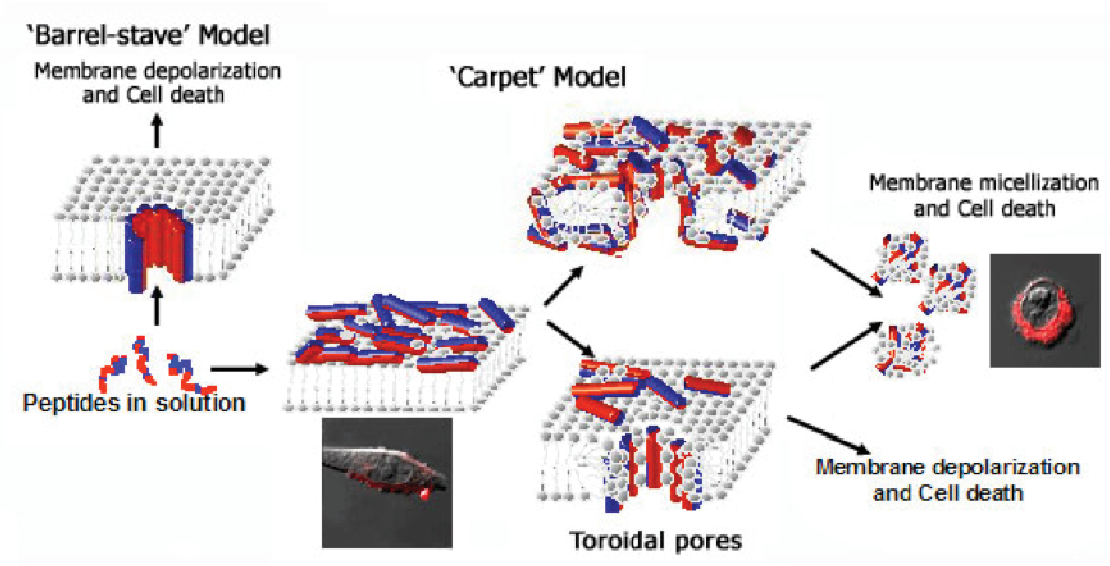

Figure 2: Model "barrel-stave", "carpet" and "toroidal pore (https://www.weizmann.ac.il/Biomolecular_Sciences/Shai/sites/Biological_Chemistry.Shai/files/carpet model.png).

At high concentration, many CAMPs have a detergent action regardless of their mechanisms at low concentration (Sato H. et al., 2006).
Another mechanism of action may involve the interaction of peptides with intracellular targets (Yeaman M.R. et al., 2003). Intracellular mechanisms of action are able to inhibit the synthesis of DNA, RNA and proteins, block the molecular chaperone, induce the production of ROS (reactive oxygen species) and inhibit breathing. These mechanisms may act independently or in synergy with membrane permeabilization events (Park SH. et al., 1998).
The antimicrobial peptides are able to interact with other molecules, whether they be conventional antibiotics or other antimicrobial peptides (Hancock RE. et al., 2000; Rosenfeld Y. et al., 2006; Scott MG. et al., 1999). CAMPs might have different isoforms with different antimicrobial activities. Production of CAMPs with similar – but not identical - structures is a strategy of natural selection to increase the spectrum of this class of antimicrobial molecules.

1.3 Pepsinogen

In recent years numerous eukaryotic proteins were described, which behave as antimicrobial peptides vectors (D'Alessio G. 2011*)*. When these proteins are in contact with proteases (bacterial and\or host), release peptides that function as CAMPs. An example is the pepsinogen; this protein is the precursor of the pepsin and it belongs to aspartic protease family. The pepsinogen is a single polypeptide chain of about 42 000 Da. It contains three disulphide bonds and has an isoelectric point of 3.7. The proteases are activated by the acid pH of the gastric lumen and have the function to digest proteins through the hydrolysis of peptide bonds. Pepsin

has a symmetry lobe and the active site is located between the two lobes (Fig.3). This enzyme cut hydrophilic or aromatic amino acid in 8 residue sequences.

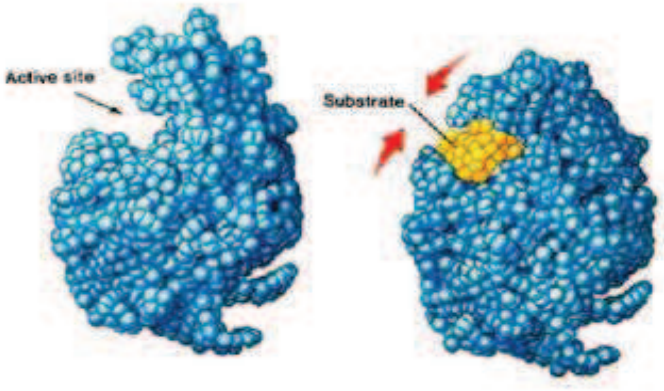

Figure 3: Active site of Pepsin
(http://readanddigest.com/wp-content/uploads/2012/11/active-site.jpg).

The amino acid sequence of pepsinogen has highlighted three regions: a signal peptide (about 15-16 aa), an activation segment (approximately 47 aa) and the active part of the enzyme (326 aa) (fig.4). The signal peptide is involved in transport mechanisms and has a highly hydrophobic sequence, which is removed during the synthesis of the pepsinogen. The activation segment (propeptide) has many basic residues and few acidic residues; on the contrary the pepsin (enzyme) is rich in acidic residues. The positive charge of lysine and arginine of the propeptide are essential to balance the negative charge of the carboxyl groups present in the pepsin. In fact, the molecule of pepsinogen is stabilized by electrostatic interactions, hydrophobic and hydrogen bonds. The activation of pepsin is obtained by breaking the peptide bond between the propeptide with pepsin (L47-V48). In this way, we have the enzyme activation and the release of the amino-terminal peptide of 47 aa (Kageyama T. 2001).

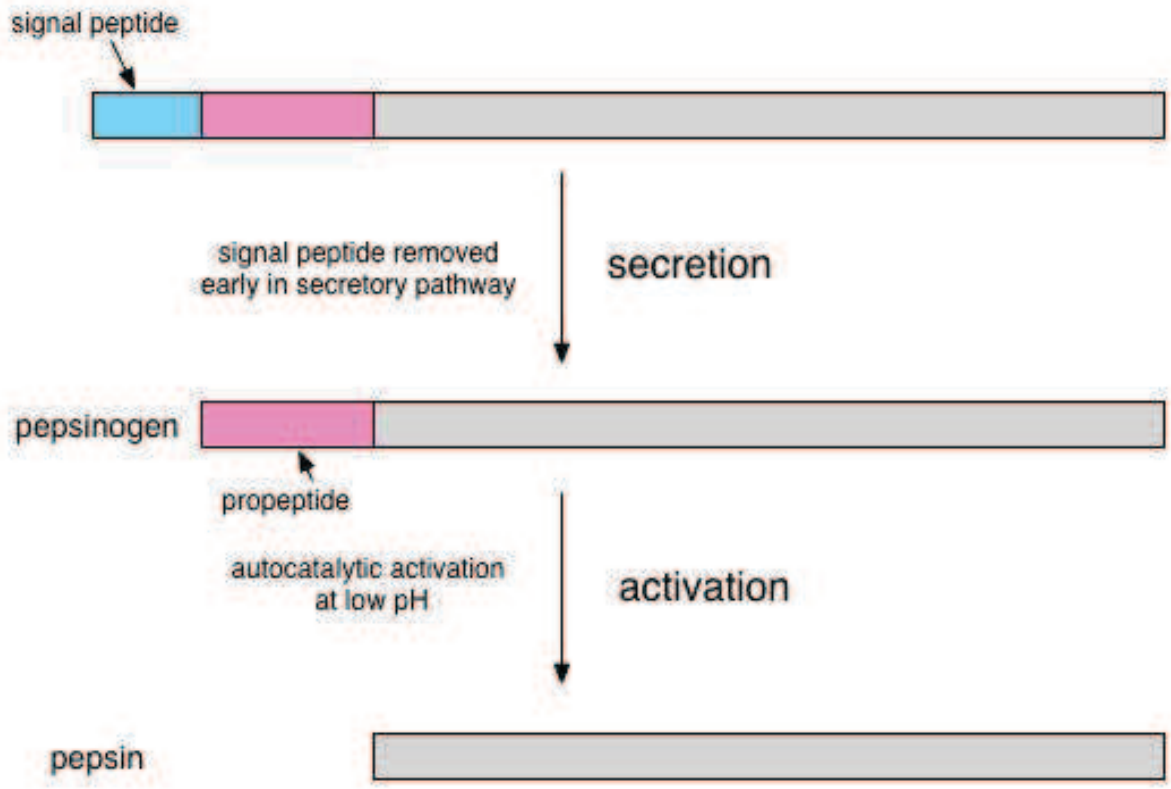

Figure 4: Pepsinogen activation
(https://i.stack.imgur.com/tx3xF.png).

There are seven different types of pepsinogen. By electrophoresis on acrylamide gel, these were numbered Pg1 to Pg7, in order of decreasing mobility. The seven members were classified into two immunologic groups: pepsinogen I (sPGI) or pepsinogen A (PGA) [comprising fractions Pg1-pg5] and pepsinogen II (sPGII) or pepsinogen C (PGC) [including the PG6-Pg7] fractions. The sPGI is exclusively produced by the peptic cells; the sPGII is produced by the glands of the whole gastric mucosa and in small quantities from the glands of Brunner located in the duodenum. Pepsinogen C is present also in the lung, pancreas and prostate. The amino acid residues present around the catalytic site are stored in different types of pepsinogen, but the amino acid sequences of the pro-peptides are different. However, they all have positive charge, crucial to the stabilization of pepsinogen. Two peptides were isolated from the stomach of Rana catesbeiana that showed a high antimicrobial activity on a broad spectrum of microorganisms (Minn I. et al., 1989). These peptides are derived from N-terminal of pepsinogens A and C. The experimental results reported by Minn, together with results obtained from prediction system of Dr Notomista E, suggest that the peptides derived from pro-sequence of pepsinogens may play a role in antimicrobial functions of the gastric mucosa of vertebrates. The pro-peptide of 47 aa presents at the N-terminal isoform A3 of Human pepsinogen (PAP 3) was produced and tested in this work.

1.4 Bacteriophage

In 1896 Hankins noted the existence of "antibacterial agents" in the Ganges (Sulakvelidze A. et al., 2001), and Jumna rivers. the first scientific experiments on bacteriophage were carried out by Felix d'Herelle (a researcher at the Pasteur Institute in Paris) in 1917. He proved the antibacterial activity of bacteriophages on *Shigella dysenteriae* in rabbits and chickens. After these preliminary results, he decided to call these antimicrobial agents phages "eaters" of bacteria. In 1930 d`Herelle founded the Institute for the Study of Bacteriophages in Georgia, where still today phages are regularly used to control bacterial infections in humans (Clokie M. et al., 2011). Bacteriophages or phages are viruses that infect bacteria. As such they are obligate intracellular parasites and can multiply only within the host bacterial cells. These prokaryotes parasites are the most abundant form of life in the biosphere. Their diversity is the result of their dynamic adaptability. When phages infect bacterial cells, they face the host antiviral defense mechanisms and consequently they need to subvert these mechanisms. Phages are classified according to their morphology, genome, and host range. The genetic material may be nucleic acid RNA or DNA and the latter can be either double-or single-stranded. The majority of phage species have an icosahedral capsid where resides the DNA; in addition to the capsid, the phage has also a collar and a tail; the tail can have the plugs and fibers (Fig.5).

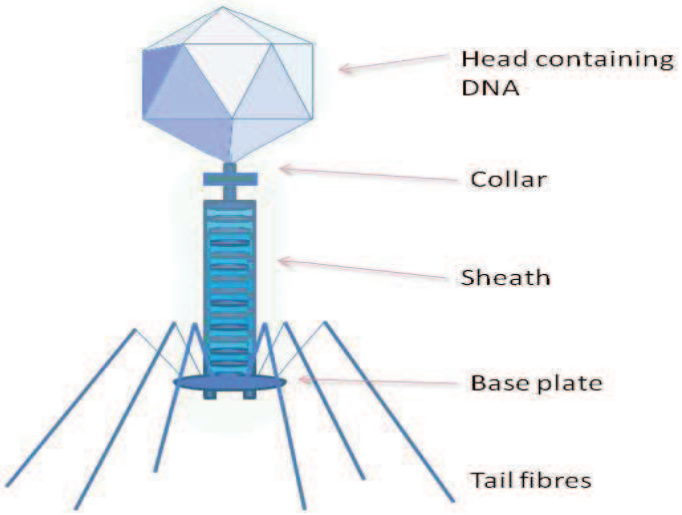

Figure 5: Bacteriophage structure (https://explorecuriocity.org/Portals/2/article%20images/Phage.png).

Bacteriophages with tail are classified into three families: *Myoviridae*, *Siphoviridae*, and *Podoviridae*, characterized respectively by a long contractile tail, a long non-contractile tail and a short tail. The bacteriophage injects his nucleic acid using a specific contraction mechanism. Once it was in
jected, the phage genome can follow two routes, the lytic or the lysogenic (Fig.6) (Clokie M. et al., 2011). In the lytic cycle, he uses the host replication system to

produce new phage particles, that are able to lyse the host bacterial cell. At the end of the lytic cycle new phages could have a "mixed" DNA, viral and in part bacterial. Upon infection, the phage “mixed” DNA is transmitted to new bacteria. When integrated in bacterial chromosome, the phage is called prophage while the bacterium is classified as "lysogenic". The prophage confers to the host bacterium immunity against phages of the same type (Adams M.H. 1959). When there are adverse conditions, the phage splits his DNA from that of the host; it produces new phage particles and begins the lytic cycle (Adams M.H. 1959).
Many viruses insert their genome into a well-defined locus of the bacterial chromosome. Upon excision phages often carry along also the bacterial genes adjacent to the insertion site (transduction). If a lytic phage infects a new bacterial host it can start the cycle again, or enter the lysogenic state.
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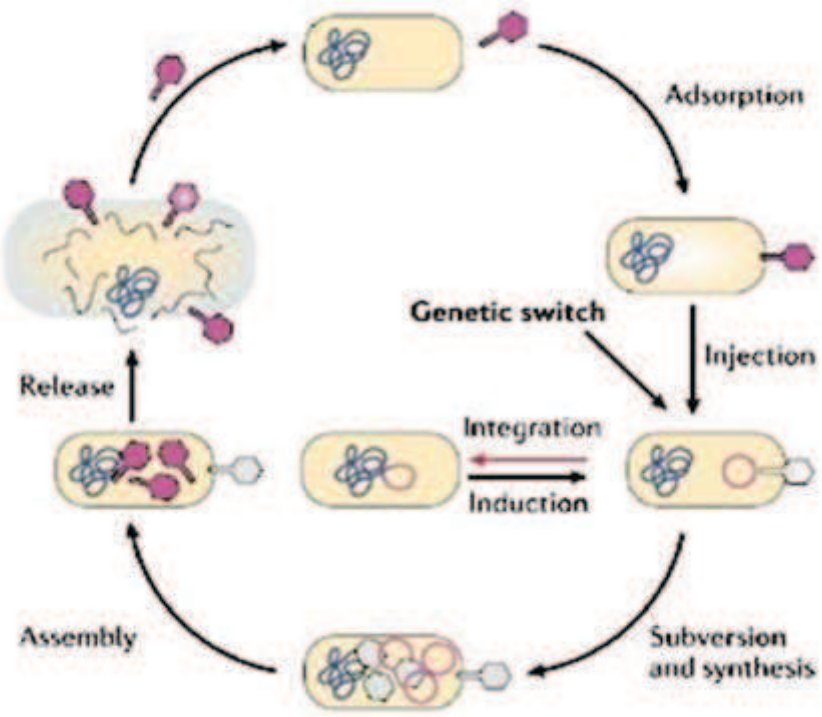

Figure 6: The lytic and lysogenic cycles of bacteriophages (http://www.nature.com/nrmicro/journal/v4/n5/images/nrmicro1393-i1.jpg).

Each bacterial species has several pathogenic strains that are not always susceptible to the selected phage. Therefore, the specificity is also a disadvantage for the phage since is required the rapid recognition of the bacterial cell by the phage. The host range (Kutter E. 2009) of the phage is restricted and limited, but this can be solved by creating a collection or pools of different phages, each capable of infecting a specific strain. Bacteriophages exclusively infect bacteria and consequently do not damage human, animal or plant organisms (Merril CR. et al., 2003). Moreover the phages do not harm the natural flora when used as an antimicrobial to control a bacterial infection in humans (Kutter EM. et al., 2015). An additional advantage is that phages

grow quickly and exponentially therefore it is possible to control bacterial infection with a single dose (Inal JM. 2003). These prokaryotes parasites increase when the microbial load is high and decrease when the bacterial concentration is low. Therefore, when the bacterial infection is eradicated phages die out**.** The in vivo proliferation favors topical application, because it allows the phage to infiltrate into the wound and reach the bacteria rapidly. The phages work fast. As a matter of fact, it was shown that the phage injected 45 minutes after infection by *Enterococcus faecium* is able to save 100% of the mice, and after one day all regain to full health *(*Biswajit B. et al., 2002*).* In addition, bacteriophages replicate at the site of infection where they are needed to lyse the pathogens, unlike antibiotics that "travel" throughout the body and do not focus on the site of infection. The identification and isolation of bacteriophages is a very simple process, fast and less expensive than the design and production of new drugs (Weber D. et al., 2000). The phenomenon of resistance in bacteria, known in many antibacterial applications, is present also in the phage therapy (Gill J. et al., 2010; Silva L. et al., 2014), but the number of bacteria that develop resistance to phage is about 10 times lower than the number of bacteria that become resistant to antibiotics *(*Sulakvelidze A. et al., 2001). Resistance may arise due to the alteration or loss of the receptors, production of modified restriction endonucleases that degrade the phage DNA, or to the inhibition of the phage intracellular development (Labrie S.J. et al., 2010). Genetic mutations affecting phage receptors represent the most frequent cause of bacterial phage resistance *(*Heller K.J. 1992; Labrie S.J. et al., 2010). The phages are constantly changing, so when bacteria become resistant, the phages evolve to infect the resistant bacteria. A disadvantage of bacteriophages is that, being obligate parasites, are not stable and have a short half-life without their host. More so, the phages can't be used to eliminate the intracellular infection because the phages are not able to enter in the eukaryotic cells. For these reasons, in this study the phages were complexed with hydroxyapatite.

1.5 Hydroxyapatite

Hydroxyapatite (HA) is a rare hexagonal mineral belonging to the apatite group, whose chemical composition is $Ca_5 (PO_4)_3 (OH)$. The nanocrystals of HA exhibit excellent biological properties, are devoid of toxicity and do not induce inflammatory response (Iafisco M. et al., 2008). In the form of salts - $CaCO_3$ (calcium carbonate), $Ca_3 (PO_4)_2$ (calcium phosphate) and CaF_2 (calcium fluoride) - HA is one of the constituents of the bones (Palazzo B. et al., 2009). Alteration of HA metabolism can lead to serious clinical consequences, such as arterial calcification, chronic kidney disease or osteoporosis (Zhu D. et al., 2012; Hofbauer LC. et al., 2007). HA is a multifunctional material, currently is used as bone void filler (Fox K. et al., 2012; Uskokovic V. et al., 2011; Dorozhkin SV. et al., 2012), and vehicle to deliver drug in eucariotic cells with well determined kinetics (Roveri N. et al., 2008; Iafisco M. et al., 2011). The hydroxyapatite, due to high affinity with proteins and other molecules, is also used in various chromatographic systems (Kandori K. et al., 2000). Different studies have also demonstrated the stability of the interaction between the nanocrystals of HA and the myoglobin /alendronate, highlighting the possibility of using an inorganic biomaterial as a support for bioactive molecules and drugs (Akawa T. et al., 1999). The biomimetic HA has a structure and composition like that of biological systems (Roveri N. et al., 2006) (Fig.7) and it has excellent properties in terms of biocompatibility, bioactivity, osteoconductivity (De Groot K. et al., 1998).

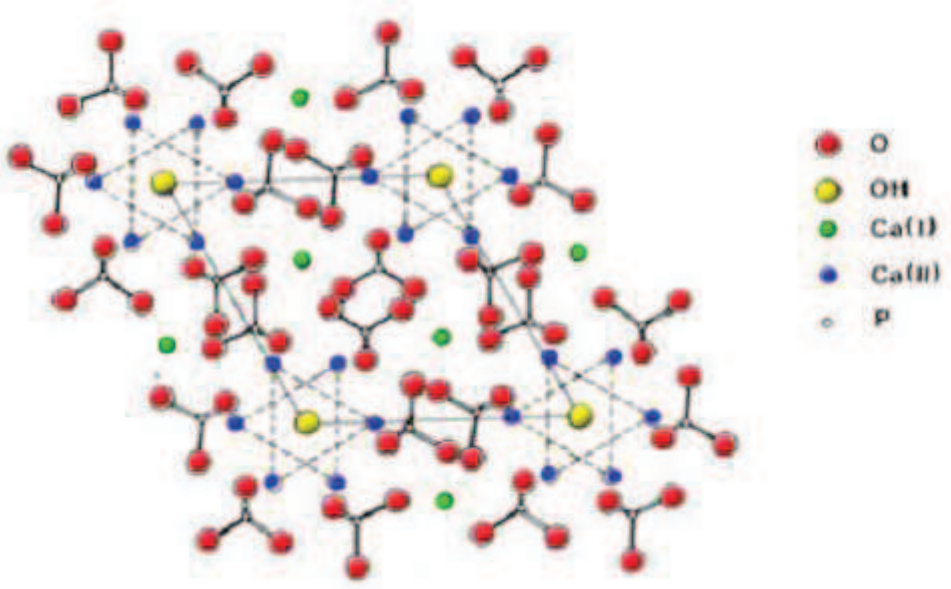

Figure 7: Hydroxyapatite structure (http://spazioinwind.libero.it/teobenedetti/ceramici_calcio_fosfati_file/image005.jpg).

The crystals have a plate-like morphology (length and width about 110 ± 5 nm and 20 ± 3 nm, respectively) and a thickness of about 8 ± 2 nm) The high reactivity of HA nanocrystals is ascribed to their amorphous surface, together with their high surface area (about 110 m^2/g), which is only slightly lower than that of biological nanocrystals (120 m^2/g). The amorphous surface of nanocrystals is responsible for the zeta potential of –20.5±1.5 mV observed at physiological pH 7.4. Exciting applications of HA in the fields of bone tissue engineering and orthopaedic therapies have already been achieved (Roveri N. et al., 2006). Dimension, porosity, morphology and surface properties are the characteristics that need to be optimized in order to adapt biomimetic HA to a specific application (Tampieri A. et al., 2005). There is evidence that nanotechnology can sensibly improve the biological responses of HA (Barroug A. et al., 2003). In literature there are many works where the silicon was substituted by HA; and this biomaterial has shown to enhance the repair rate of bone tissue (Thian ES. et al., 2006; Porter AE. et al., 2004; Patel N. et al., 2002). Furthermore, the hydroxyapatite reduces the adhesion of bacteria (Chen Y. et al., 2010; Rameshbabu N. et al., 2007; Kim TN. et al., 1998). The presence of bioactive molecules on the surface of biomimetic HA nanocrystals makes it possible to transfer information and selectively influence the biological environment. The interaction between proteins and different kinds of inorganic surfaces, such as HA nanocrystals, carbonates and phosphate, plays an important role in many applications - including medicine, pharmacy, nanodevices, biosensors, and bioengineering (latour RA. 2008; Dee KC. et al., 2003). Biomimetic represents an important tool for the design and synthesis of innovative materials and devices (Mann S. 1997; Sarikaya M. and Aksay I. 1995; Bensatude VB. et al., 2002; Sanchez C et al., 2005), which offer a unique approach to overcome many shortcomings in materials science (Molle P. et al., 2005). In this study, hydroxyapatite was used to deliver the phages into eukaryotic cells. Furthermore with hydroxyapatite the phages can be stabilized and protected

when used as ingredient in food production or incorporated into the packaging system.

1.6 Food Packaging

The packaging has the role to protect the food from contamination by pathogenic agents and environmental factors (oxygen, moisture and heat), prolonging the shelf life of the product and maintaining the nutritional and organoleptic characteristics. Following the growing demand by consumers of natural and safe food (Kerry JP. et al., 2006) researchers have directed their attention to improve the quality and food safety. The most significant innovations in this area took place in the early twentieth century, when plastic, polyethylene and propylene have replaced the metal, glass and paper used for packaging and food storage (Wilson C. et al., 2007). In the past, the packaging systems functioned only as passive barriers for food protection from the environment, while the modern "Active packaging" system interacts with the food through the release of antimicrobial and antioxidant compounds (Brody A. et al., 2001; Lopez-Rubio A. et al., 2004). Examples of natural molecules with antimicrobial activity are: essential oils derived from plants (such as basil, thyme, oregano, cinnamon and rosemary), enzymes derived from animals (for example, lysozyme, lactoferrin), bacteriocin produced by microbes (nisin), organic acids (propionic acid, citric acid) and natural polymers (chitosan) (Brewer R. et al., 2012; Lopez-Pedemonte TJ. et al., 2003, No. HK et al., 2007). The presence of antimicrobials in food packaging, as well as extending the shelf life of the food, reduces deterioration of the product, and the occurrence of food borne illnesses associated with microbial contamination (Brody AL. et al., 2008). It is very important to eliminate from the food the bacteria that can cause food poisoning (Ricke SC. 2003). Oils of clove, oregano, rosemary, thyme, sage, and vanillin are very effective (Skandamis PN. et al., 2002) against bacteria; this antimicrobial activity is due to the presence of phenolic functional groups, such as hydroxyl groups (Dorman HJD. et al., 2000). Other inhibitory agents against Gram-positive and Gram-negative bacteria (Mangena T. et al., 1999; Marino M. et al., 2001) are bacteriocin. They are small bacterial peptides that show strong antimicrobial activity against closely related bacteria. Nisin is a polypeptide produced by *Lactococcus lactis spp*, approved as a food additive with GRAS status in over 50 countries worldwide. It has a broad spectrum of activity against various lactic acid bacteria and other Gram-positive bacteria. Moreover, the effect of nisin can be enhanced by using exposure to chelating agents, osmotic shock and freezing, because these treatments make the cell wall of Gram-negative microorganisms more permeable and therefore more susceptible to the nisin (Galvez A. et al., 2007). The enzymes represent another group of natural compounds used in food packaging. Lysozyme for example, is a lytic enzyme found in foods, such as milk and eggs; which can hydrolyze β-1,4linkages between N-acetyl muramic acid and N-acetylglucosamine (Cunningham FE. et al., 1991). In industrialized countries, infections transmitted by food are a major public health problem, and about half of the episodes of disease in humans are caused by salmonella. In fact, this bacterium is one of the main agents of food disease transmission in humans. The transmission of Salmonella to humans occurs through ingestion of contaminated food of animal or vegetable origin. Despite controls and monitoring carried out by the authorities, the cases of Salmonella infection are very numerous. For this reason, it is very important to look for an alternative packaging system, which has as its primary objective to reduce the presence of salmonella in the food.

1.7 Salmonella

In 1884 Georg Theodor August Gaffky isolated the agent of typhoid; after two years Daniel E. Salmon and Theobald Smith isolated the *Salmonella choleraesuis* from pigs. The genus Salmonella belongs to the family *Enterobacteriaceae*; which are rod-shaped organisms, Gram negative, asporogenous, equipped with flagella, aerobic-anaerobic facultative, catalase positive, oxidase negative (Fig.8). The *Enterobacteriaceae* family includes many kinds of health interest such as *Escherichia, Shigella, Citrobacter, Klebsiella, Enterobacter*, and many others. Within the genus *Salmonella* exist a large number of serotypes, distinct for the different composition of the somatic and flagellar antigens. Kauffmann in 1931 introduced the classification scheme of salmonella, accepted around the world and it is still in use. The genus *Salmonella* is divided into two species, *S. enterica and S. bongori.* The species enterica is in turn divided into six subspecies: *enterica, salamae, arizonae, diarizonae, houtenae and indica*. Today there are more than 2,400 serotypes of enteric species but the human infections are caused by a limited number of serovars (Grimont P.A.D. et al., 1997).

In industrialized countries, infections transmitted by food are a major public health problem; the *Salmonella* and *Campylobacter* are the main agents of food illness.

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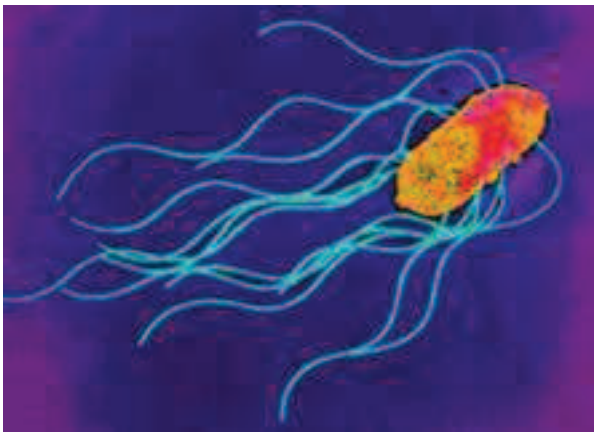

Figure 8: *Salmonella (http://www.journaldelenvironnement.net/mediatheque/4/7/9/000008974_5.jpg).*

Salmonella enterica serovar *Enteritidis* and serovar *Typhimurium* are the most common serovars isolated during outbreaks of foodborne in the United States and European Union *(*Finstad S. et al., 2012).

Epidemics are frequent and can be trans-national, when products are distributed worldwide. The salmonellosis is subject to surveillance. The European Community legislation (Directive 99/2003 / EC, 2160/2003 / EC Regulation) has included it in the compulsory activities for the different States members. The Institute of Health coordinates numerous laboratories of the Italian Health Service to control of the Salmonella. The salmonella infection is spread by faecal-oral route through ingestion of contaminated food or drinks (Beuchat L.R. et al., 2002). The incubation period is very short; in fact, the symptoms of the disease can occur even 12 hours after ingestion of the bacterium. Once the ingestion of the microorganism is occurred, the

development of symptomatic infection depends on the number of ingested bacteria (the minimum infective dose is hypothesized between 10^2 and 10^3 cells), but can vary in the different serotypes and also depending on the host conditions. Young, elderly or people with reduced immune defences are more affected by salmonellosis. The symptoms of the gastrointestinal tract are (Tauxe RV. et al., 1998):

- Abdominal pain;
- Nausea and vomit;
- Fever and diarrhea (stools of a strong dark green colour);
- General malaise.

The pathogenesis of Salmonella infection is complex and multifactorial. The bacterium colonizes the intestine, invades the intestinal mucosa and stimulates the migration of polymorphonuclear leukocytes (PMN) with diarrhea induction.
In the very young or immunocompromised people, the infection may spread from the intestines and become systemic (Darwin KH. et al., 1999). Salmonella has several virulence factors, necessary to implement all stages of infection (Wallis TS. et al., 2000) including defence systems that allow survival in acidic pH environments, useful to overcome the gastric barrier (Slauch J. et al., 1997); factors involved in the colonization of the intestine, allowing the bacterium to effectively adhere to the intestinal lumen cells (fimbriae type 1 and 3) (BaumL er AJ. et al., 1997) and factors that allow to cross the intestinal epithelium or to survive in macrophages (PhoP enzymes and PhoQ) (Gunn JS. et al., 2000). Data from the Ministry of Health show that there are about 10-15,000 cases of non-typhoid salmonellosis each year and 50% of food infection are given by salmonella. *Salmonella enterica* serovar *rissen* was recognized as one of the most common serovar among humans and pork production systems in different parts of the world, especially Asia (Pornsukarom S. et al., 2015).

2. AIM OF THE PROJECT

The development of antibiotics in the 1928 has significantly contributed to reduce the mortality rate for human diseases. Now, nearly 90 years later, we are in the "post-antibiotics" era in which several bacterial infections can't be successfully treated because even our most powerful drugs are become useless. Infective outbreaks caused by resistant bacteria are numerous, the most known is the global pandemic of methicillin-resistant *Staphylococcus aureus* (MRSA) infection (Moran GJ. et al., 2006). The US health care system invests $34 billion each year to eradicate this pathogen (Filice GA. et al., 2010) that is more lethal than pathology as HIV/AIDS, Parkinson's disease, and emphysema (Klevens RM. et al., 2007). In the United States, a huge number of antibiotics is used each year, but only half of this is provided to people. The other half is used in agriculture or in the farms as growth promotants in food and in the animals (Levy SB. et al., 2002). This inappropriate use contributes to the increase in antibiotic resistance and the phenomenon affects both gram negative and gram positive bacteria. The development of new antibiotic is very slow due to market failure and regulatory disincentives. The Antibiotics resistance represented a serious problem for the Pharmaceutical industries because according to these phenomena many bacteria are able to developed the resistance to antibiotics making their use increasingly less effective. An antibacterial alternative could be the use of bacteriophages and antimicrobial peptides. In this study, we propose the possibility to use these antimicrobial agents against bacterial infection, in the pharmaceutical field as new therapy and in the agro-food field to reduce the use of synthetic preservatives and simultaneously increase the shelf-life of products. Moreover we provide a comprehensive picture regarding their advantages and disadvantages, the mode of action, specificity and safety.

3. MATERIALS AND METHODS

3.1 PAP3 identification

To quickly identify cryptic antimicrobial peptides, Dr. Notomista E. (Department of biology, University of Naples Federico II) has developed a system of prediction to correlate structural characteristics of CAMPs with their antimicrobial properties (Minimal inhibitory concentration). The prediction system was used to analyze human secreted proteins deposited into databases. The N-terminal isoforms A3, A4 and A5 of human pepsinogen identified as potential CAMPs. These sequences were aligned and the N-terminal isoform A3 was chosen.

3.1.1 Expression of the recombinant protein

The cells made competent (100 µl) with $CaCl_2$ were transformed using 20 ng of the recombinant plasmids pET22b(+)/ONC-DCless-His/PAP3 (provided to us by Dr. Notomista E.). To produce the heat shock, the competent cells were incubated on ice for 60 minutes and after were incubated at 42℃ for 3 minutes. Then 1 ml of LB was added to cells and then the sample was incubated at 37℃; after incubation, the cells were harvested at 4000 rpm at room temperature by centrifugation for 5 minutes; The precipitated cells were suspended in 100 µl of LB. The cells were plated on LB agar in the presence of ampicillin 100 g/mL and after were incubated at 37℃ for 20 h. Subsequently 10 colonies were picked from the plate, and they were inoculated into 10 mL of LB containing ampicillin 100 µg/mL. The cells were incubated at 37℃ for 2h until OD 0.6-0.7 nm/mL. The expression of the recombinant protein was induced by addition of IPTG to final concentration of 0.4 mM. The induction was carried out by incubating the cells at 37℃ for 3 h. Like negative control, one samples has not been induced. After the induction phase, the cells were harvested by centrifugation at 12000 rpm for 1 min at room temperature.

3.1.2 Cell lysis and treatment of inclusion bodies

The cells were suspended in Tris-HCl 0.1 M pH 7.4 and 20 mM EDTA and were lysed by sonication for 30 min. The soluble fraction of the cell lysate was separated from the insoluble fraction by centrifugation at 12000 rpm for 30 minutes at 4℃. The components of cell membranes were removed by treatment with detergents and denaturants such as Triton X-100 2%, Urea 2 M EDTA and 20 Mm. The inclusion bodies were denatured and reduced with Guanidine-HCl 6 M and 10 mM β-mercaptoethanol. The sample was centrifuged at 12000 rpm at 4 ℃ for 10 minutes and the supernatant was recovered and was subjected to the next step of purification by IMAC.

3.1.3 Chromatographic IMAC

The chromatographic resin was constituted from a hydrophilic matrix of agar to 6% (cross-linked) covalently bound to a chelating group. This chelating agent was loaded with Ni2 + ions, in this way the resin binds the histidine residues.
5 mL of resin were centrifuged at 3500 rpm at 4℃ for 15 minutes to remove the ethanol 20%. After the resin was washed 3 times with 30 ml of the equilibrium buffer then it was centrifuged at 3500 rpm at 4℃ for 15 minutes and incubated with the sample for 20h at 4 °C. The elution step was carried out into column, through the use of sodium acetate at pH 5.0 containing 0.1 M guanidine-HCl 6 M. The eluate was

collected in fractions of 1 ml and the absorption was measured at 280 nm for each fraction.

3.1.4 Determination of protein concentration

The concentration of proteins was performed using the Bradford assay. This method is very fast, cheap and sensitive. It is based on the use of the Coomassie brilliant blue G250 (CBBG) that binds to residues of arginine, tryptophan, tyrosine, histidine and phenylalanine. In acidic solution the binding of the dye Coomassie Brilliant Blue G-250 to proteins causes the formation of a blue product which has an absorption maximum at 595 nm (Bradford LW. 1976). The intensity of the absorption at 595 nm is proportional to the protein concentration. To determine the concentration of the samples was constructed a calibration line of bovine serum albumin (BSA). The samples were prepared in a final volume of 1 mL containing 500 µl of reagent and 500 µl of water and absorbance was evaluated at 595 nm.

3.1.5 Acid hydrolysis

The denatured proteins in guanidine buffer/6M HCl were dialyzed in 0.1 M acetic acid to pH 3. At the end of dialysis, the samples were centrifuged at 12000 rpm at 4℃ for 30 min and the supernatant was subjected to acid hydrolysis. This method consists of three steps:

- Acidification to pH 2 by adding HCl;
- Incubation at 60℃ for 24 h;
- Neutralization to pH 7.2-7.4 by the addition of NH3.

Subsequently the sample was centrifuged at 12000 rpm for 60 minutes at 4℃. In this way the carrier precipitates while the peptide remains in solution. The degree of purification was analysed by SDS-PAGE 20%.

3.1.6 SDS-PAGE

In summary the elements that make up the electrophoretic system are:
Resolving gel is polyacrylamide solution to 15-20% that separates proteins according to their molecular weight. To polymerize 5 mL of Resolving were added 5 µl of TEMED (catalyst) and 50 µl of ammonium perosolfato 10 mg/mL (APS, radical initiator). Upper gel is an acrylamide solution (6%) this serves to position the samples on a same line and create the space in which samples are loaded. To polymerize the Upper gel (2.5 ml) were added 5 µl of TEMED and APS 25 µl of 10 mg/mL. The electrophoretic run was performed at 150 V for 60 minutes, using the running buffer Tris/Glycine 1X. After the electrophoretic run the gel was stained using of a Coomassie blue 0.2% solution (w/v) in acetic acid at 10% (w/v) and 25% isopropyl alcohol (p/v). Subsequently the discolouration was made in an acetic acid solution to 7% and ethanol 25%.

3.1.7 HPLC chromatography

The chromatographic separation was performed with a C18 column (column size 250 x 4.6 mm; diameter of the spheres 5 µm; pore size of 280-360 Å). The analyses was conducted at a flowrate of 1 ml/min by performing elution gradient with two buffers: trifluoroacetic acid (TFA) 0.05% in water (buffer A); 0.05% trifluoroacetic acid in acetonitrile (buffer B). The analyses were performed using the following gradient: 1) 10 minute isocratic elution at 5% buffer B; 2) phase of elution in a linear gradient from 5 to 20% of buffer B in 5 minutes; 3) elution in a linear gradient from 20 to 30%

of buffer B in 40 minutes; 4) Final washing of the column with 95% of buffer B. The spectrum of the eluted peaks was recorded between 210 and 600 nm.
The samples to be analysed were prepared in a total volume of 2 ml in the presence of 5% acetonitrile and 0.05% TFA.

3.2 PAP3 antimicrobial activity

The bacteria (*Salmonella typhimurium, Salmonella rissen, Listeria monocytogenes, Staphylococcus aureus* and *Escherichia coli*) were grown in Muller Hinton (MH) at 37℃ until exponential phase. The samples were then centrifuged at 8000 g for 10 minutes, washed with saline, and suspended in broth MH at approximately 10^6 CFU/mL; 60 µL of inoculums were placed in a multi-well polystyrene plate from 96 wells (bacterial concentration of 10^5 CFU/mL). PAP3 dissolved in AMAC was tested at different concentrations. The plate was incubated at 37℃ for 20 hours (Romanelli A. et al., 2011). Microbial growth was measured using a microplate reader model 680 (Biorad, Hercules, CA) to 600 nm (Capparelli R. et al., 2005). The MIC value (Minimal inhibitory concentration) was obtained using the following formula: [(ODcontrol-ODblank)-(ODactivity-ODblank)/(ODcontrol-ODblank)] x 100.

3.3 Phage isolation

The bacteriophages were isolated from different bacterial strains: *Salmonella typhimurium, Salmonella rissen, Listeria monocytogenes, Staphylococcus aureus* and *Escherichia coli*. Gram-positive bacteria were grown in TS broth whereas Gram-negative bacteria instead in LB broth at 37℃. When cultures reached the exponential growth phase, mitomycin C (1 µg/mL) was added. After 30 min the samples were centrifuged and bacteria were washed with LB or TS broth and incubated again for 4 h at 37℃. The supernatants were filtered through a 0.22-µm membrane and screened for the presence of phages, using the spot test (Adam MH. et al., 1959). Individual plaques were expanded in 2 ml of broth containing the sensitive bacterial host (10^6 CFU/mL).

3.3.1 Phage host range

Gram-negative bacteria were cultured in LB broth while Gram-positive bacteria were grown in TS broth at 37℃ until exponential phase. After 500 µl of each bacterial strain was individually poured into a tube containing 4 ml of 0,7% top agar. The suspension was transferred to a Petri dish with LB agar or TS agar layer and solidified. Then 10 µl of phage was spotted on lawns of different bacterial strains and the plates were incubated at 37℃ overnight, subseq uently the spectrum of infection was recorded.

3.3.2 MOI test

MOI of phage (Multiplicity of Infection) was determined using bacteria cultures grown overnight in MH. The required bacterial concentration (10^4 CFU/mL) was obtained by tenfold serial dilutions in 0.1% peptone water. The MOI range used in this test was between $1*10^{-3}$ to 1000, serial 1:10 dilutions of the phage were performed in SM buffer. The wells of 96 well microplate were filled with 100 µl of bacterium and 100 µl of the diluted phage. The wells of positive and negative control contained respectively 100 µl of strain plus 100 µl of SM buffer and 100 µl of MH plus 100 µl of SM buffer. Bacterial counts were performed in triplicate for each condition tested.

The plate was incubated at 37℃ for 18h then the optical density was measured at OD 600nm.

3.3.3 One step growth curve

An exponential culture (10 ml) of bacterium was centrifuged at 13800 g for 5 min at 4℃ and the pellet was suspended in 5 ml of LB (Gram-negative) or TSB (Gram-positive). The phages were added at a MOI 1000 and allowed to adsorb for 5 min at RT. Then the mixture was centrifuged and the pellet was suspended in 10 ml of LB or TS broth and incubated at 37℃. The titer of phages was measured using the double-layer agar technique. Aliquots (1,5 ml each) were collected at 5 min intervals over a time period of 90 min. Samples were diluted and were plated to determinate the latent and the rise period.

3.3.4 Electron microscopic analysis

Bacteria (10^8 cells/mL) in LB (Sigma, USA) were distributed in 24-well plate and incubated overnight at 37℃. After the surnatant of each well was eliminated and the wells were washed with PBS. The bacteria attached to the wells were fixed with 1,25% of glutaraldehyde, 2% of paraformaldehyde and 0,1 M NaCacodylate buffer ON at 4℃. Following the dehydration with the ethanol 70% for 1h, 80% for 1h, 90% for 1h, 95% for 1h and 100% for 1h, the wells were cut and they were attached on the support for SEM.

The phage stock (40 ml), containing approximately 10^8 PFU/mL, was purified on a CsCl density gradient by ultracentrifugation, and dialyzed against SM buffer at 4℃ overnight. Phage particles were negatively stained with 2% phosphotungstic acid (PH 7, 2) for 5 min (the excess was removed with a filter paper). Phages were observed in a Philips EM 300 electron microscope.

3.3.5 Phage DNA extraction

Bacteriophage DNA was isolated using phenol-chloroform method. Briefly, the lysate (40 ml) with 10^8 PFU/mL was centrifuged at 51,000 x g for 2 h after, the pellet was suspended in 1 ml of PBS. The phage suspension was treated with 12.5 mM $MgCl_2$ (Acros Organics, NJ, USA), 0.8 U/mL of DNase (Sigma, USA), and 0.1 µg/mL of RNase (Sigma, USA) (final concentrations) and incubated at room temperature for 1 h to eliminate bacterial DNA (Nale JY. et al., 2015). Following the addition of 0.5% sodium dodecyl sulphate (Sigma, USA), 20 mM of EDTA (Sigma, USA) and 0.5 mg/mL proteinase K (Fisher Scientific, Germany), it was incubated at 56℃ for 2h. Phage DNA was extracted with an equal volume of phenol-chloroform (1:1 v/v) after the sample was centrifuged at 15,000 x g for 5 min. Afterwards the aqueous layer was treated with 0.3 M sodium acetate (Sigma, USA) and 2 volumes of ice-cold ethanol. After placing on ice for 10 min, the sample was centrifuged at 21,000 X g for 20 min, and the pellet was dissolved in 5 Mm Tris HCl. The DNA concentration was analysed using a NanoDrop ND 1000 spectrophotometer and the integrity was checked using a 0,7% agar gel.

3.3.6 Phage DNA sequencing

DNA sequencing was performed using the Ion Torrent PGM platform, yielding a total of 2,199,543 reads (660Mb) and an average coverage of ~13,200x. Quality control and trimming were carried out using in-house-implemented Python scripts, assembly was performed using the SPAdes software (Bankevich A. et al., 2012), and finishing was completed using the DraftDr software (version 1.0 CRS4; M. Orsini, unpublished data). Genome annotation was manually curated after a preliminary annotation performed by RAST (Aziz KR. et al., 2008).

3.4 Hydroxyapatite

The nanocrystals of biomimetic hydroxyapatite (HA) [$Ca_5(PO_4)_3(OH)$] were precipitated from an aqueous solution of $(CH_3COO)_2Ca$ by slow addition of H_3PO_4, keeping the pH constant at 10 by addition of $(NH_4)OH$ solution (Palazzo B. et al., 2009). To produce HA with the stoichiometric Ca/P ratio (1.67), the Ca $(CH_3COO)_2$ suspension and the H_3PO_4 solution used were 83 and 50 mM, respectively.
The reaction mixture was stirred at 37℃ for 24 h, after the mixture was left in static condition for 2h to allow deposition of the inorganic phase. This was repeatedly washed with water and freeze-dried at - 60℃ under vacuum (3 mbar) for 12 hours.

3.4.1 Transmission electron microscopy

The analysis were carried out using a 1200 EX microscope, linked to X-ray analysis detectors and a 3010 UHR operating at 300 kV (JEOL Ltd, Tokyo, Japan). Few droplets of the samples (in ultrapure water) are been deposited on perforated carbon foils supported on conventional copper microgrids.

3.4.2 Zeta potential analysis

To determinate the Zeta potential was used a Coulter Delsa 440, this instrument is able to measure the electrophoretic velocity of particles, checking the Doppler shift of scattered laser light simultaneously at four different scattering angles: 7.5, 15.0, 22.5 and 30.0. For this test was used 0.05 g $l^{-1}$of hydroxyapatite in 10^{-2} M KNO3.

3.4.3 Determination of surface area

Measurements were done using a Sorpty 1750 instrument (Carlo Erba) using N2 absorption at 77 K (Brunauer S. et al., 1938).

3.5 Complex (phage-*hydroxyapatite)*

The complex was prepared by mixing the hydroxyapatite (100 mg/mL) with the phages (10^8 PFU/mL) in a ratio 1: 1 v/v. The mixture was maintained in agitation at RT for 30', 90',180',300' and overnight. Then the complex was centrifuged and the pellet suspended in SM buffer. The amount of phage adsorbed on hydroxyapatite was determined using the double agar overlay method. This purpose were prepared 5 aliquots constituted by 1 ml of hydroxyapatite (100 mg/mL) and 1 ml of phages (10^8 PFU/mL). At each time interval one aliquot was centrifuged at 5000 rpm for 5 ' and the pellet was suspended in 1 ml of SM buffer. Serial dilutions were performed for each sample and after the dilutions were tested using the spot test 500 µl of *Salmonella rissen* were added to 4 ml of Soft Agar 0.7% on LB agar plate and subsequently were made three spotts from 10 µl of each dilution. The plates were incubated at 37℃ and after 24 h the lysis plaques were counted.

3.5.1 Study of the complex

To estimate the stability of the phage and the complex in the time, we determined the titer using the double agar overlay method, by plating a dilution series 10^{-1}–10^{-11}. The titer was evaluated immediately after the phage and the complex preparation and at 7 days intervals for about 2 months. During this time, the samples were stored at +4℃ (Mattila S. et al., 2015).

The effect of an acidic and an alkaline pH on phage and the complex was evaluate. A total of 100 μl of phage free or phage with hydroxyapatite was added to test tubes containing 900 μl of SM buffer (pH 2, pH 4, pH 7 and pH10). After 24 h of incubation at 37 ℃, the percentage of phages able to lyse the host bacterial cells was estimated (Jurczak-Kurek A. et al., 2016).

3.6 Cytotoxicity trials

3.6.1 MTT assay

The human cells used in this study were the HepG2 (liver carcinoma). These were cultured in MEM (Minimal Essential Media), 10% fetal bovine serum, 2mM Glutamine + 1% Non-Essential Amino Acids (NEAA) + 100 IU/mL penicillin, and 100 μg/mL streptomycin (all from Gibco, Paisley, Scotland). The cells (30000 cells/well) were grown in 96 well plates in volumes of 200 μl of medium and they were treated respectively with the PAP3 (200 μg/mL), the phage (10^8 PFU/mL), the complex (10^8 PFU/mL) and hydroxyapatite (100 mg/mL) for 24h, 48h and 72h. The wells of positive and negative control contained respectively 10% DMSO *a*nd PBS. The test was performed in triplicate for each condition tested. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium was dissolved in PBS, at a concentration of 5 mg/mL and a volume of 20 μl added to each well. After 2 hours, the medium was aspirated from the wells, 150 μl of isopropanol was added and the plate was incubated at 37℃ for 30`. Then the optic al density was measured at 560 nm.

3.6.2 LDH assay

A lactate dehydrogenase (LDH) assay was performed using a CytoTox 96 Non-Radio cytotoxicity assay kit (Promega, Madison, WI, USA) at 24 h, 48h and 72h.

3.7 Intracellular killing activity

The liver cancer cells (HepG2) were distributed in 24-well plates (10^6 cells/well), incubated overnight (37℃, 5% CO_2) in Minimal Essential Media (MEM) supplemented with 10% fetal calf serum (all from Gibco, Paisley, Scotland). Subsequently one part of the cells were infected with *Salmonella rissen* (10^4 CFU/mL) for 1h. Then the medium was removed, and the cells were washed with PBS and incubated in MEM containing 10% fetal calf serum and gentamicin (12,5 μg/mL) at 37℃ in 5% CO_2 (Capparelli R. et al., 2007). After 3 hours the cells were treated with the phage (10^8 PFU/mL), complex (10^8 PFU/mL) and hydroxyapatite (100 mg/mL) overnight. The other part of the cells were infected with *Salmonella rissen* (10^4 CFU/mL) and at the same time treated with the phage (10^8 PFU/mL), the complex (10^8 PFU/mL) and the hydroxyapatite (100 mg/mL) for 1 h. Then the cells were treated with gentamicin like above. After 3 h the medium was removed, and the cells were incubated in MEM containing 10% fetal calf serum overnight. The cells of both experiments were lysed with Tween 20 (final concentration, 0.05 %) and each

lysate was then serially diluted in PBS and plated onto XLT4. The plates were incubated at 37℃ and after 24 h typical Salmonella colonies were counted.

3.8 Production of fluorescent complex

The fluorescent complex was prepared mixing 1 ml of the complex (10^8 PFU/mL) with 10 micro liter of fluorescein (Sigma, USA) (6 µg/ml) overnight in agitation. Subsequently the mix was centrifuged at 5000 rpm for 1 min and the pellet was suspended in SM buffer.

3.8.1 Internalization of fluorescent complex

The HepG2 cell line was cultured at 37℃ and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 100 units/mL each of penicillin and streptomycin and 2 mmol/L glutamine. Cells were plated on polylysine-coated slides in 24 multi-well plates at a density of 1.0 x 10^5 per well. After 24h, cells were exposed to different concentrations of fluorescent complex (10 µL of 1:1000, 10 µL of 1:100, 10 µL of 1:10) for different time (2, 5 and 24 h). The control cells were treated with 10 µL of SM buffer. After treatment, the medium was removed from each well, cells were rinsed twice with PBS, fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature. DAPI was used to counterstain nuclei. Slides were observed using a Zeiss LSM 710 Laser Scanning Microscope (Calr Zeiss MicroImaging GmbH). Samples were vertically scanned from the bottom of the coverslip with a 63 x or 40 x (1.40 NA) Plan-Apochromat oil-immersion objective. Images were generated with Zeiss ZEN Confocal Software (Calr Zeiss MicroImaging GmbH).

3.9 Bacterial reduction assay on meat

A Single colony of *Salmonella rissen* was amplified in Luria-Bertani (LB) broth (Oxoid) at 37℃, harvested in exponential growth ph ase. The bacterial concentration required for the experiment was prepared by serial dilutions in 0.1% peptone water (PW) (Anuraj T. et al., 2015). For the test, a sample of the meat 250 g was divided into 5 equal parts, of which 4 were infected with 20 ml of *Salmonella rissen* (10^3 CFU/mL) and incubated at room temperature for a correct bacterial attachment, while the fifth was treated only with 5 ml of PBS (negative control). After 30 minutes, 3 of the four infected parts were treated respectively with the phage (10^8 PFU/mL), the complex (10^8 PFU/mL) and hydroxyapatite (100 mg/mL), while the fourth has not undergone any treatment (positive control). All samples were stored at 4℃ for 7 days and analysed at regular intervals of 24h. For each time interval were taken 5 g from each sample and homogenized in 45 ml of solution 0.1% PW at 200 rpm for 1 min, using the Stomacher 400 Circulator, Seward Ltd. Subsequently, 100 µl of sample were plated on XLT4 agar and incubated at 37℃ for 24h. The efficacy of each treatment was evaluated by counting the CFU/mL.

4. RESULTS

4.1 Identification and production of PAP3

The prediction system was used to analyze human secreted proteins archived into databases. The N-terminal isoforms A3, A4 and A5 of human pepsinogen were identified as potential CAMPs. The alignment of N-terminal sequences of these isoforms has shown that the three peptides differed for one single amino acid: the isoform A3 had a lysine residue (K43), while the A4 and A5 isoforms had a residue of aspartic acid (E43). Therefore, the A3 isoform of human pepsinogen (PAP3) had two positive charges compared to A4 and A5 isoforms. This feature made the isoform A3 particularly interesting for this study.

PAP3 was expressed in E. coli strain BL21DE3 using the plasmid pET22b(+)/ONC-DCless-His/PAP3 (provided by Dr. Notomista E.). The cells were induced with IPTG and with SDS-PAGE 15% was proved that induced cells had expressed more recombinant protein than not induced cells (Fig.9).

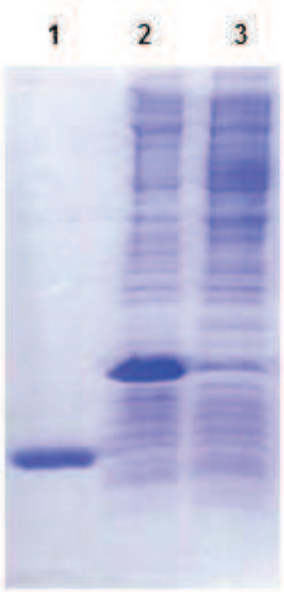

Figure 9: Electrophoretic analysis on SDS-PAGE15%. Lane 1: Lysozyme (14,3 kDa); Lane 2: Induced cells; Lane 3: Uninduced cells.

To verify that the recombinant protein was expressed as inclusion bodies, an aliquot of the culture was subjected to lysis by sonication and the fractions (soluble and insoluble) were separated by centrifugation and were analyzed on SDS-PAGE 15%. The recombinant protein was expressed only in insoluble form as inclusion bodies. This fraction was treated with detergents to remove E. coli proteins, subsequently the sample was analyzed on SDS-PAGE 15%. The recombinant protein was fully recovered while the contaminating proteins derived from E. coli were significantly reduced. The sample was treated with Guanidine- B-mercaptoethanol and the soluble fraction was subjected to purification by affinity chromatography IMAC. The eluate was collected and the yield of the purification process (assessed by Bradford assay) was approximately 80%. The acid hydrolysis of protein ONC-DCless-His/PAP3 was performed at pH 2 at 60℃. At the end of hydrolysis step, the sample

was analyzed on SDS-PAGE 20%, protein ONC-DCless-His/Pap3 was hydrolyzed with an efficiency of approximately 90-95%, releasing PAP3.
The peptide purification was realized taking advantage of the different solubility properties between the onconase and PAP3 at different pH. The soluble and insoluble fractions were analyzed on SDS-PAGE at 20%, the carrier was present only in the insoluble fraction while the peptide was present only in that soluble (Fig.10).

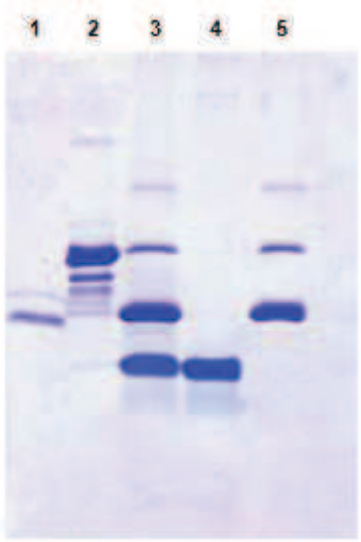

Figure 10: Electrophoretic analysis on SDS-PAGE 20%. Lane 1: Lysozyme; Lane 2: ONC-DCless-His/PAP3 protein before hydrolysis; Lane 3: ONC-DCless-His/PAP3 protein after hydrolysis; Lane 4: Soluble fraction; Lane 5: Insoluble fraction.

The PAP3 peptide purified by selective precipitation at pH 7 was analyzed by reverse phase chromatography on C18 column. The chromatogram showed the presence of a main peak eluted at 52.28 min. The electrophoretic analysis of this peak proved that it was PAP3. The protein concentration was determined by UV spectrometry in this way it was possible to estimate that by 100 mg of fusion protein ONC-DCless-His/PAP3 was obtained about 30 mg of PAP3 peptide.

4.2 PAP3 antimicrobial activity

Antimicrobial tests carried out on several bacterial species, such as *Salmonella typhimurium*, *Salmonella rissen*, *Listeria monocytogenes, Staphylococcus aureus* and *Escherichia coli* have demonstrated that the pepsinogen at the concentration of 220 µg/mL inhibits 90%-100% of bacteria (tab.1). These data provide evidence that the PAP3 (as already predicted by bioinformatic tools) is a CAMP effective against Gram-negative and Gram-positive bacteria.

Table 1: Antimicrobial activity of PAP3.

Bacteria	**PAP3 200 µg/mL**
Staphylococcus aureus	95%
Listeria monocytogenes	93%
Salmonella rissen	100%
Salmonella typhimurium	100%
Escherichia coli	97%

4.3 Isolation of phages

Following incubation with mitomycin (1 µg/mL for 30 min), the phages were successfully isolated from all strains used in this study (*Salmonella typhimurium*, *Salmonella rissen*, *Listeria monocytogenes,* and *Escherichia coli).* The phage Φ1 isolated by *Salmonella rissen* (Fig.11), was chosen for further studies in view of its higher lytic activity in vitro and larger host range.

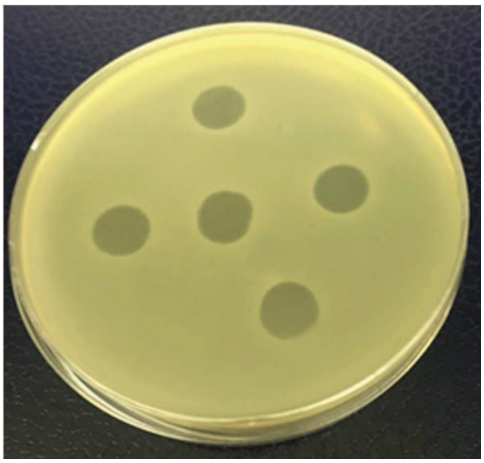

Figure 11: Lysis plaques of phage Φ1.

4.3.1 Host range

The infectivity of phage Φ1 was investigated using fourteen other Salmonella species: *S.livingstone, S.infantis, S.potsdam, S.thompson, S.mbandaka, S.winston, S.montevideo, S.virchow, S.ohio, S.jerusalem, S.inganda, S.wil, S. typhimurium, S.enteritidis*. All these strains were isolated from food at the Institute Zooprofilattico Sperimentale Del Mezzogiorno. Eleven of the tested strains were susceptible to phage Φ1 (tab.2).

Table 2: Phage Φ1 host range determination on different Salmonella strains.

Bacteria	Lytic activity
S.livingstone	+
S.infantis	+
S.potsdam	+
S.thompson	+
S.mbandaka	+
S.winston	+
S.montevideo	-
S.virchow	+
S.ohio	+
S.jerusalem	-
S.inganda	+
S.wil	+
S.typhimurium	-
S.enteritidis	+

- , Negative lysis result; + , positive lysis result.

4.3.2 MOI test

The ability of phage Φ1 to reduce the growth of bacteria *Salmonella rissen* was assessed using the phage at MOI from $1*10^{-3}$ to 1000 (serial 1:10 dilutions of the phage were performed in SM buffer). A decrease in OD_{600nm} between the wells with bacteria and the wells with bacteria plus phages were observed for all the MOI tested. Bacterial growth inhibition was proportional to the phage dilution (Fig.12). The best MOI was 1000 but also to 0,001 there was a significant inhibition of bacterial growth.

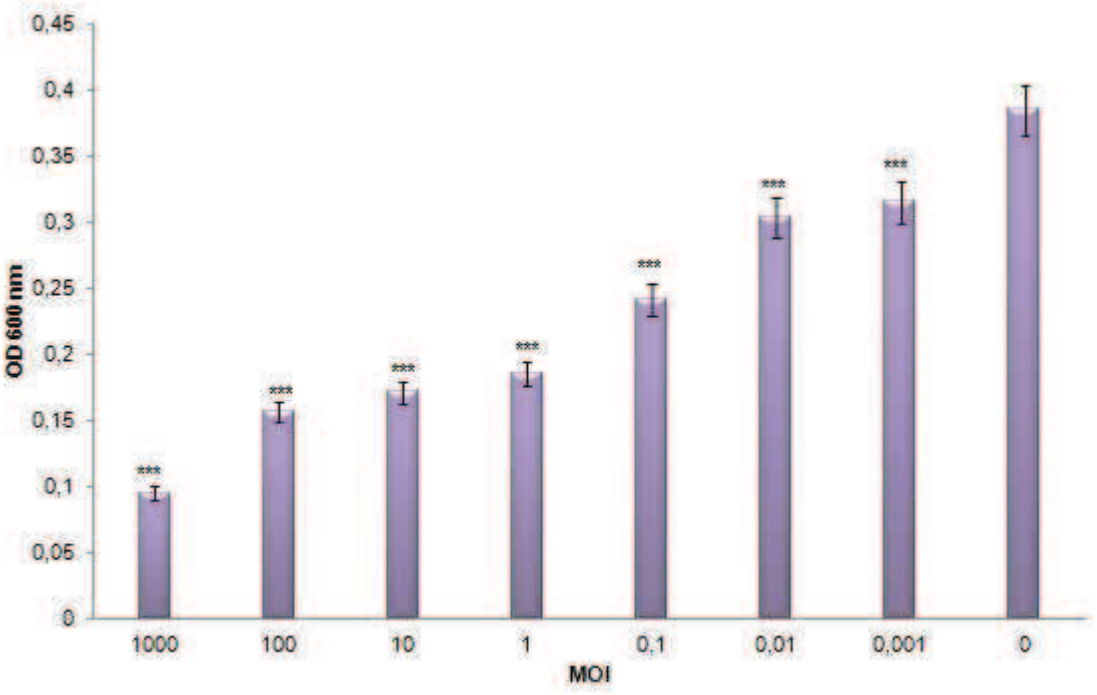

Figure 12: Representation of phage activity on a growing of *Salmonella rissen.* The absorbance is greatly reduced when MOI is 1000 compared to control (MOI 0). *** p<0,001. *Each value are the mean ± DS of 3 independent experiments. Statistical analysis was performed with Student's t tests.*

4.3.3 One step growth curve

The one-step growth curve of phage Φ1 was determined, taking sample aliquots each 5 min intervals over a time period of 90 min. In this way were calculated the latent phase , the rise phase (Fig.13) and the burst size. They were 30 min, 55 min, respectively and the burst size was of 54 phage particles.

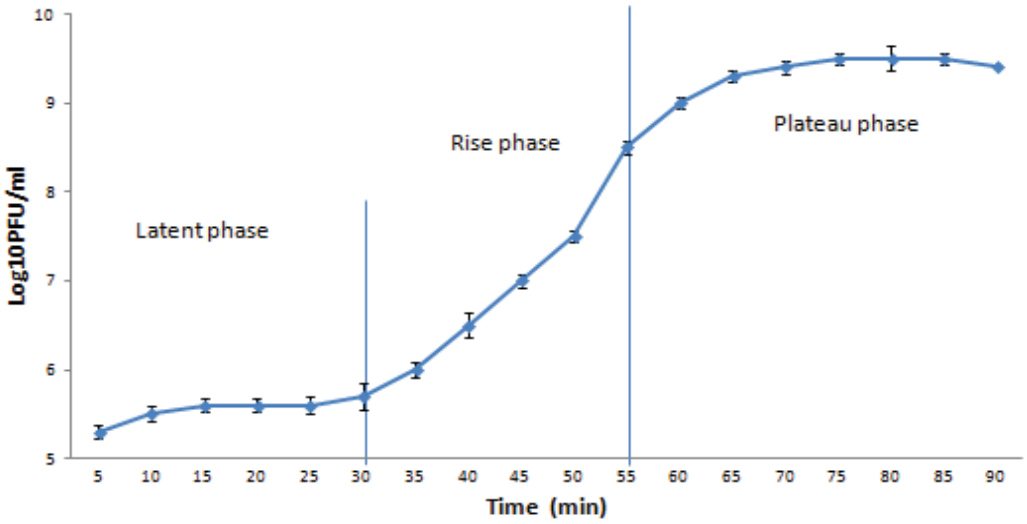

Figure 13: One step growth curve of phage Φ1. Each value is the mean ± DS of 3 independent experiments with 3 replicates each. Statistical analysis was performed with Student's t tests.

4.3.4 Electron microscopic analysis

The phage Φ1 had the head diameter of 57 ± 1, 0 nm and the length of the flexible tail was 16 ± 2, 0 nm. The total length of phage was 73 ± 2, 0 nm. The electron microscope characteristics indicated that the phage of the *Salmonella rissen* (Fig.14) belonged to the *Podoviridae* family (Fig.15). Into the phage capside there is the nucleic acid, it could be RNA or DNA. The phage Φ1 had DNA.

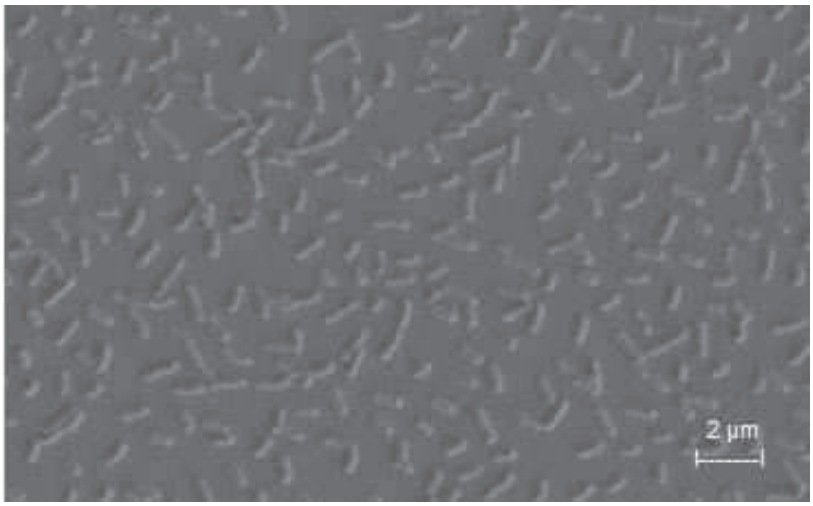

Figure 14: *Salmonella rissen* observed with a SEM. Scale bars, 2 µm.

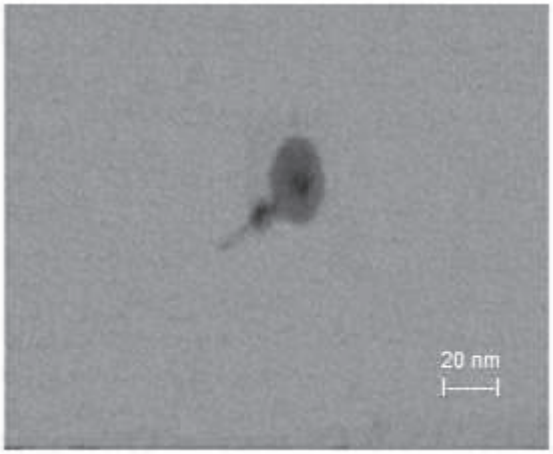

Figure 15: Phage Φ1 observed with a TEM.
Scale bars, 20 nm.

4.3.5 Phage DNA sequencing

DNA phage was extracted by phenol:chlorofom method. Using the Nanodrop the DNA quantity and quality were analysed. The concentration was 560 ng/µl, the purity was evaluated analysing the ratio 260/280 and 260/230. They were 1,76 nm and 1,70 nm respectively. The integrity was checked using a 0,7% agarose gel (Fig.16).

1 Lane: DNA phage
2 Lane: EasyLadder I

Figure 16: DNA phage extracted using Phenol:Chloroform method

DNA of phage Φ1 was sequenced using the Ion Torrent PGM platform and assembly was performed using the SPAdes software. Genome annotation was manually curated after a preliminary annotation performed by RAST. The genome consisted of 42,990 bp (Fig.17), with a G+C content of 48.6%. The genome contained 65 predicted coding sequences (CDSs). Of those CDSs, including 7 tail shaft, 1 tail fiber, 1 coat, 1 terminase, 1 portal, 22 other phage like protein. The remaining 32 CDSs encoded hypothetical proteins. No genes associated with lysogenize, toxin production, Salmonella virulence, or antibiotic resistance were identified, therefore suggesting a possible use of this phage as a prophylactic agent for the control of Salmonella. Further analysis, however, will be required to assign potential functions to the several unidentified and hypothetical gene.

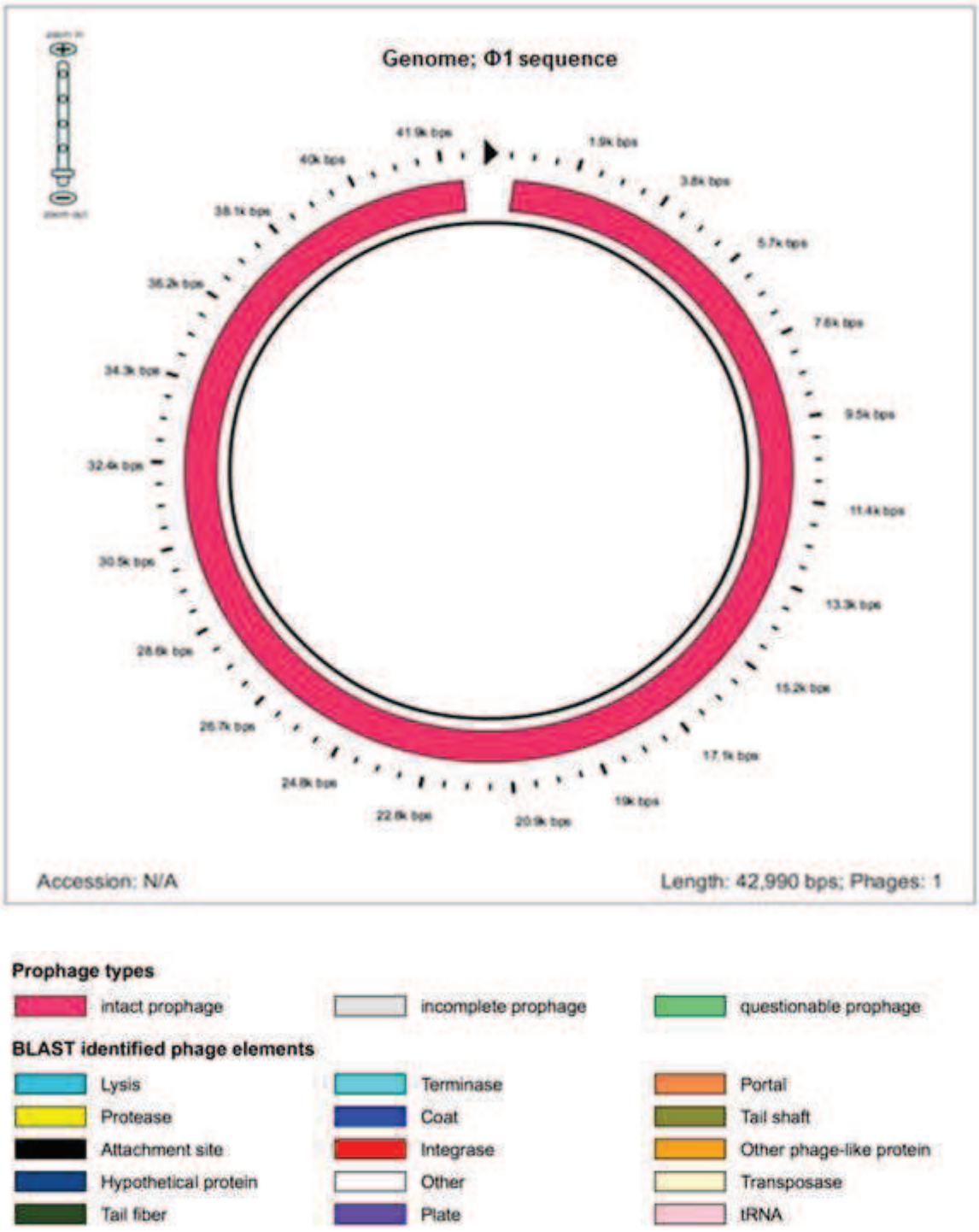

Figure 17: Phage Φ1 genome representation. PHAST (PHAge Search Tool) is a web server designed to rapidly and accurately identify, annotate and graphically display prophage sequences within bacterial genomes or plasmids. Our results show that the genome investigated was catalogued like an intact prophage.

Through Blast analysis was confirmed that the phage Φ1 belonged to the *Podoviridae* family and showed 52.3% identity (determined with EMBOSS stretcher) to phage PHAGE_Salmon_vB_SosS_Oslo_NC_018279 it was therefore classified as a T4-like phage. According to the genome structure of T4 phages (*Miller E. et al., 2003),* the bacteriophage exhibits a genome functionally divided into three parts: preearly, early, and late regions. The preearly region includes a large terminally repeated sequence. The early genes are involved in phage metabolism, DNA replication, and lysis, also including a tRNA gene cluster. The late genes encode structural proteins for mature phage particles (Fig.18).

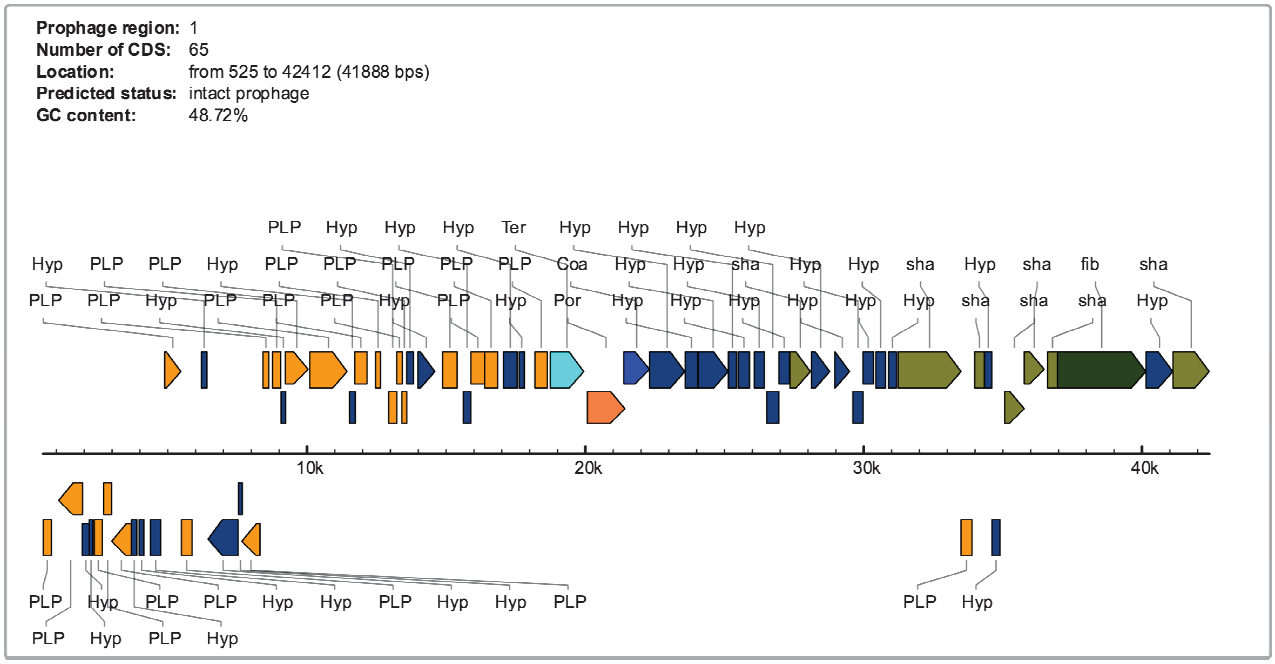

Figure 18: Graphic representation of gene annotation on the phage sequenced.

4.4 *Hydroxyapatite*

The biomimetic hydroxyapatite produced in this study appears to be similar to that found in the human body. It reveals a plate-like morphology (Fig.19), with length, width and thickness of about 110 ± 5 nm, 20 ± 3 nm, and 8 ± 2 nm respectively. The high reactivity of hydroxyapatite is ascribed to its amorphous surface, and to high surface area about 110 m^2/g, which is only slightly lower than that of biological nanocrystals (120 m^2/g). When the HA nanocrystals are at pH 7.4 they shown a zeta potential of 20.5±1.5 Mv, this value is ascribed to their amorphous surface.

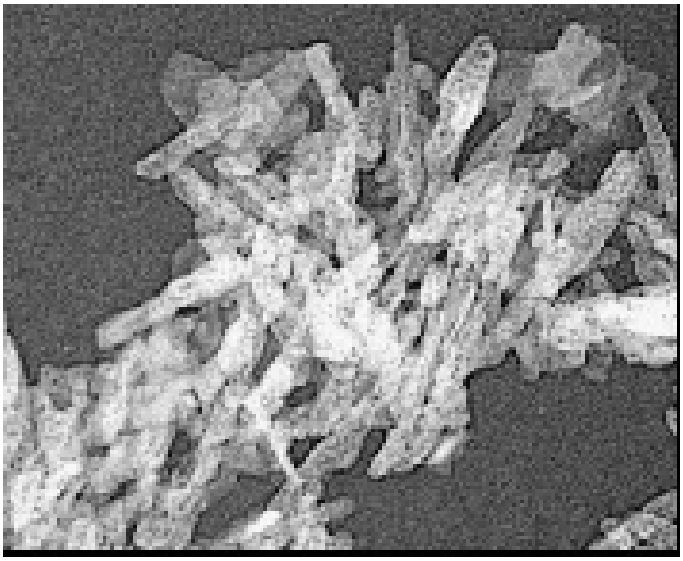

Figure 19: Transmission electron microscope of the biomimetic hydroxyapatite.

4.5 The complex (phage-hydroxyapatite)

The complex was produced mixing the hydroxyapatite (100 mg/mL) with the phages (10^8 PFU/mL) in agitation. The amount of phages attached on hydroxyapatite was detected after 30', 90',180',300' and overnight using the spot test. The results have shown that the phages was attached to hydroxyapatite already after 30', but only after 24h all the phage in solution was absorbed. (Fig.20).

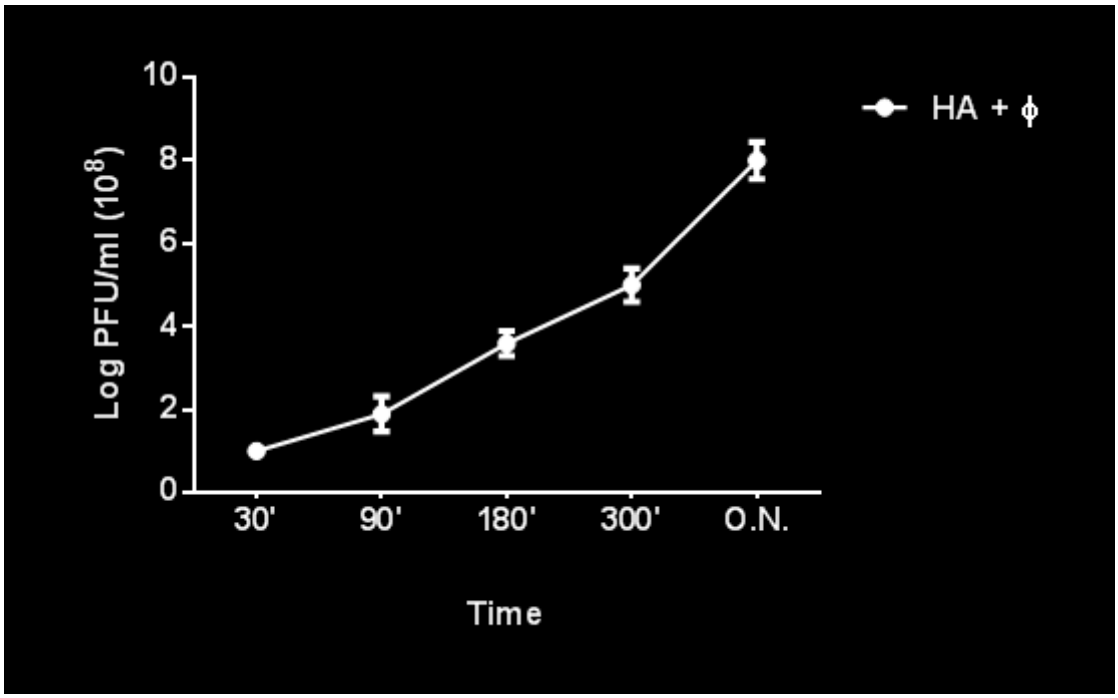

Figure 20: The absorbing of the phages on the hydroxyapatite after different time of the incubation (30', 90',180',300' and overnight). Each value is the mean ± DS of 3 independent experiments with 3 replicates each. Statistical analysis was performed with Student's t tests.

4.5.1 Study of the complex

The stability of the phage and the complex was determined doing dilution series and spotting them on the double agar overlay. The titer was estimated immediately after the preparation and each 7 days for about 2 months. During this time, the samples were stored at +4℃. The title (PFU/mL) of phage af ter 6 weeks was decreased while the title of the complex was always the same (Fig.21).

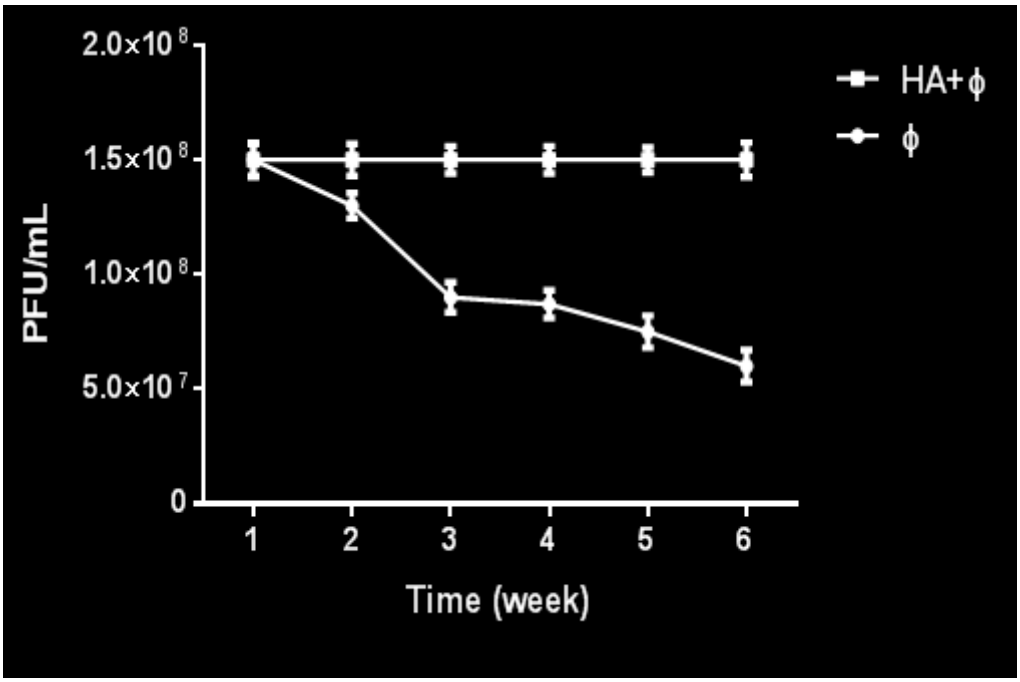

Figure 21: The stability of the phage and the complex in the time. Each value is the mean ± DS of 3 independent experiments with 3 replicates each. Statistical analysis was performed with Student's t tests.

The hydroxyapatite did not alter the specificity of the phage, in fact the complex had lysate 11/14 salmonella strains analysed as phage alone.
Phage and the complex were tested to evaluate the effect of an acidic and an alkaline environments on their vitality, the results showed that phage alone was extremely sensitive to pH 2 while the complex remained stable (Fig.22).

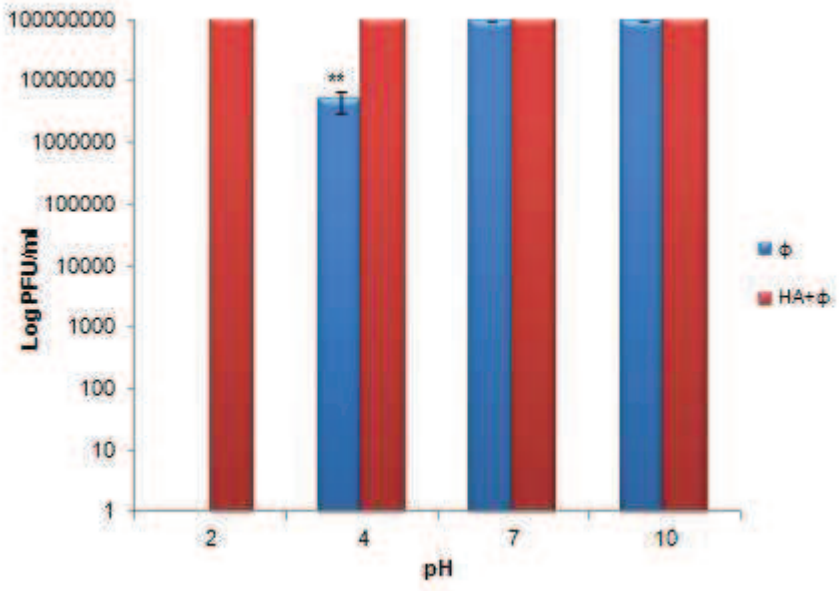

Figure 22: Effects of pH on the stability of phage and complex. ** p<0,01. Each value is the mean ± DS of 3 independent experiments with 3 replicates each. Statistical analysis was performed with Student's t tests.

4.6 Cytotoxicity trials

The human cells HepG2 (liver carcinoma) were treated respectively with PAP3 (220 µg/mL), phage (10^8 PFU/mL), complex (10^8 PFU/mL) and hydroxyapatite (100 mg/mL). Using the MTT assay was possible to evaluate that PAP3 was toxic to eukaryotic cells, while the other treatments did not affect the cell viability: the cells were viable after 72 hours of treatment. (Tab.3). In addition, the LDH enzyme (released into the medium as sign of necrotic cell death) *(Awad WA. et al., 2012),* was evaluated after 24h, 48h and 72h by treatments, using the CytoTox 96 Non-Radio cytotoxicity assay kit (Promega, Madison, WI, USA). All treatments have induced low production of LDH (Lactate dehydrogenase) for up to 72 hours, except for PAP3 which resulted a significant increase of this enzyme (Fig.23).

Table 3: MTT test on cells HepG2 (liver cancer cells). The cells treated respectively with PAP3 (220 µg/mL), the phage (10^8 PFU/mL), the complex (10^8 PFU/mL) and hydroxyapatite (100 mg/mL) for 24h, 48h or 72h. Each value is the mean of three independent experiments.

Time (h)	ϕ	HA	ϕ+HA	PAP3
24	97%	100%	98%	51%
48	94%	98%	96%	43%
72	92%	98%	94%	38%

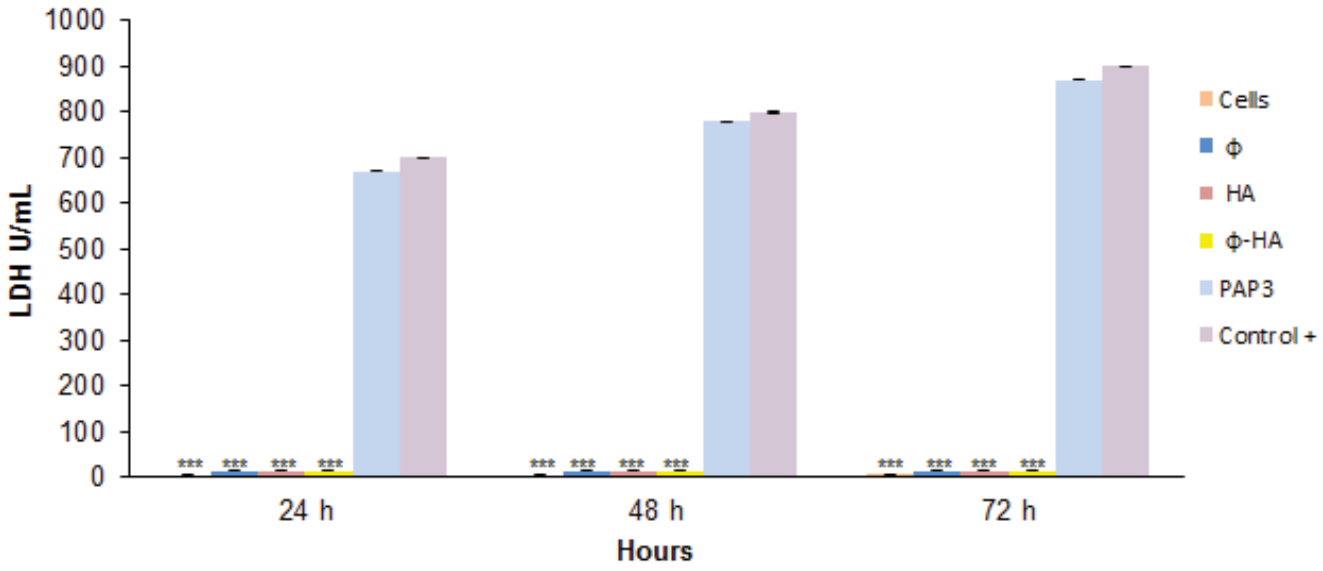

Figure 23: LDH production in HepG2 cells treated respectively with PAP3 (220 µg/mL), phage (10^8 PFU/mL), complex (10^8 PFU/mL) and hydroxyapatite (100 mg/mL) for 24h, 48h, 72h. Positive control: control plus LDH provided by the kit. Each value is the mean ± DS of 3 independent experiments with 3 replicates each.

4.7 Intracellular killing activity

To evaluate the intracellular killing activity, we have done two experiments using the liver cancer cells (HepG2). In the first experiment, the cells were infected with *S. rissen* and after treated with gentamicin (to kill the extracellular bacteria), the phage (10^8 PFU/mL), the complex (10^8 PFU/mL) and hydroxyapatite (100mg/mL), were added to the wells individually. In the second experiment the cells were infected with *Salmonella rissen* and treated with phage (10^8 PFU/mL), complex (10^8 PFU/mL) and hydroxyapatite (100mg/mL) at the same time. Then the cells were treated with gentamicin like above. For both experiments, the positive control was represented by Salmonella rissen infected cells. Following the incubation (24h) the cells of both experiments were lysed and the number of intracellular bacteria was determined by plating of the lysate on XLT4 agar. In the first experiment (Fig.24 A) only the complex killed intracellular bacteria while in second experiment (Fig.24 B) (where the bacteria infection was done at the same time of the treatments) the phage and the complex have reduced the growth of Salmonella. These results demonstrate that phage could not penetrate inside eukaryotic cells and only when delivered inside cells by hydroxyapatite or bacteria (in this case *Salmonella rissen*), it was able to kill intracellular bacteria efficiently.

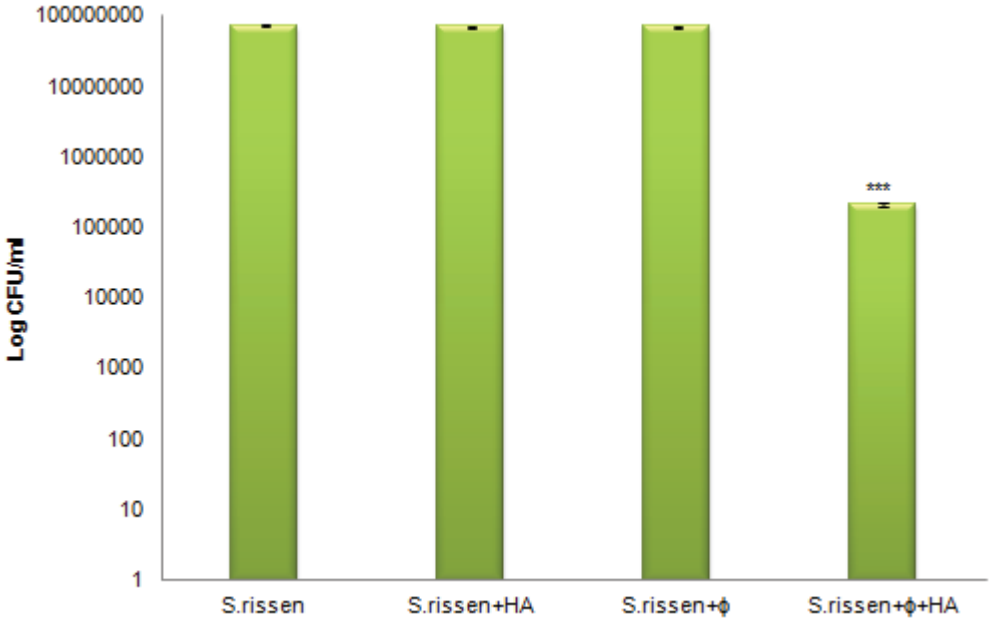

Figure 24 A: Intracellular killing activity (first experiment). The HepG2 cells were infected with *Salmonella rissen* (10^4 CFU/mL) and after were treated respectively with the phage (10^8 PFU/mL), the complex (10^8 PFU/mL) and hydroxyapatite (100 mg/mL) for 24h. Positive control was represented by *Salmonella rissen* infected cells. *** $p<0,001$. Each value is the mean ± DS of 3 independent experiments with 3 replicates each. Statistical analysis was performed with Student's t tests.

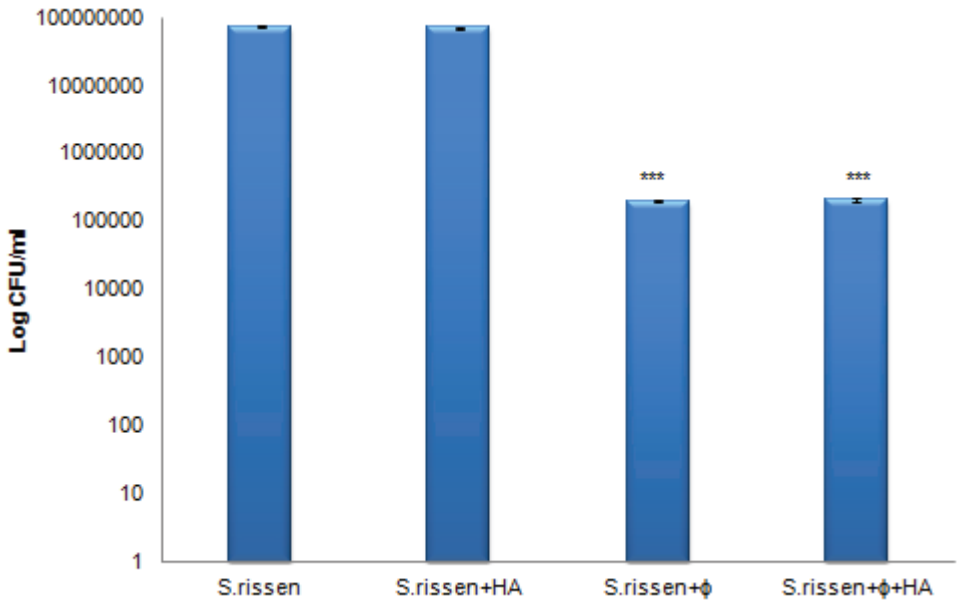

Figure 24 B: Intracellular killing activity (second experiment). The HepG2 cells were infected with *Salmonella rissen* (10^4 CFU/mL) and treated with phage (10^8 PFU/mL), complex (10^8 PFU/mL) and hydroxyapatite (100 mg/mL) at the same time. Positive control was represented by *Salmonella rissen* infected cells.*** p<0,001. Each value is the mean ± DS of 3 independent experiments with 3 replicate each. Statistical analysis was performed with Student's t tests.

4.8 Internalization of fluorescent complex

In order to demonstrate the internalization of complex in HepG2 cells we visualized the green luminescence of the fluorescent complex by Laser scanning confocal microscopy. Figure 25 showed that numerous green fluorescence of complex were internalized in the cytoplasm of the HepG2 cells already after 2h of treatment with 10 µl of 1:1000, with an accumulation of luminescent particles after the overnight treatment. Nuclei were counterstained with DAPI dye (blue). No fluorescent light was detected in the control cells.

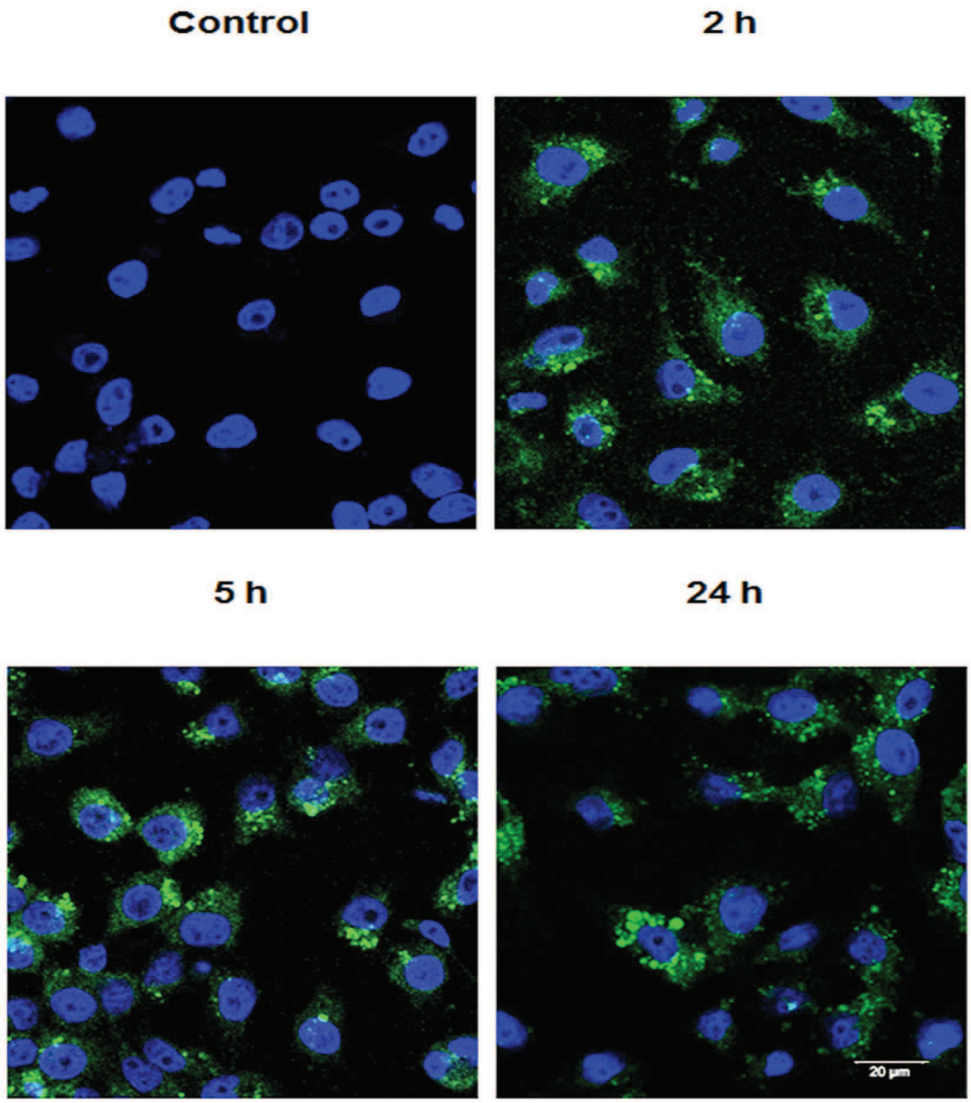

Figure 25: Confocal microscopy images of the cells control and the cells treated with the complex at 2h, 5h, 24h. Scale bars, 20 µm.

4.9 Bacterial reduction assay on meat

The data in Figure 26 show the reduction of Salmonella contamination on the meat after treatment with the phage (10^8 PFU/mL), the complex (10^8 PFU/mL) and the hydroxyapatite (100 mg/mL). The colonies of Salmonella in the sample treated with the phage were reduced by 0.3 log CFU/g while the sample treated with hydroxyapatite had the same Salmonella contamination of the positive control 5.5 log CFU/g. Instead the complex (hydroxyapatite-phage) had sensibly reduced the CFU number. In fact it was able to reduce Salmonella rissen of 3 log CFU/g.

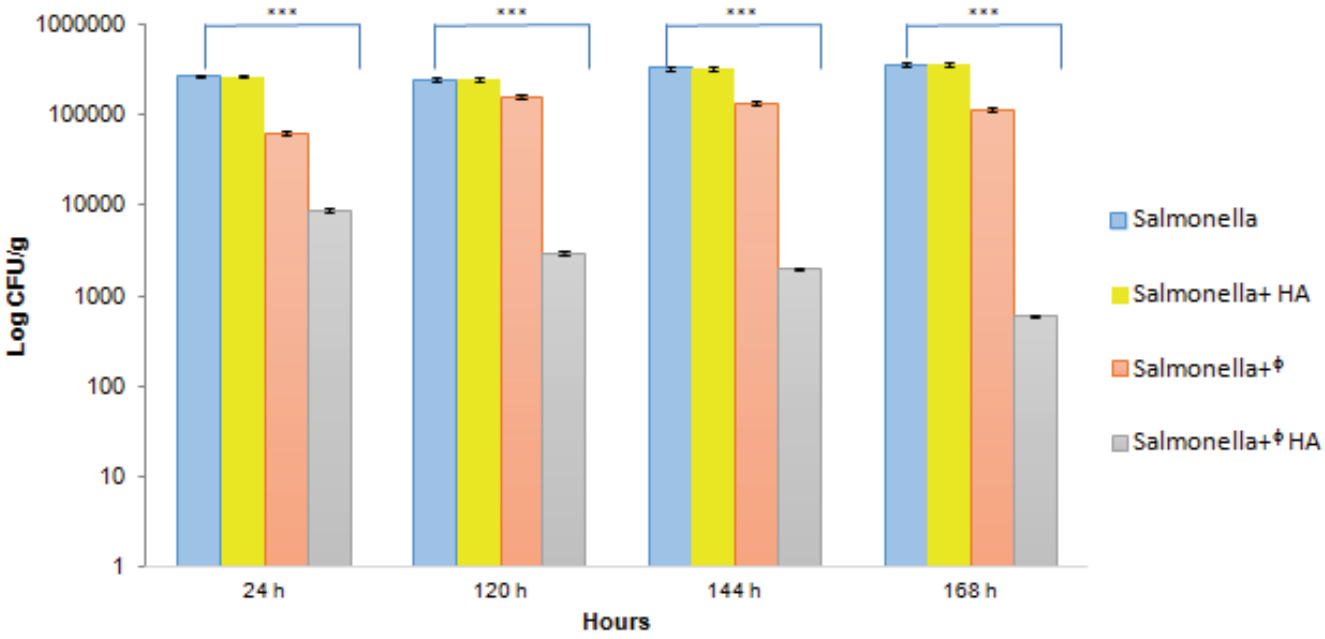

Figure 26: Bacterial reduction assay on meat. The samples of the meat were infected with *Salmonella rissen* (10^3 CFU/mL) and were treated respectively with the phage (10^8 PFU/mL), the complex (10^8 PFU/mL) and hydroxyapatite (100 mg/mL). Positive control was represented by *Salmonella rissen* infected meat. *** p<0,001. Each value is the mean ± DS of 3 independent experiments with 3 replicates each. Statistical analysis was performed with Student's t tests.

5. DISCUSSION

The inappropriate and unnecessary antibiotic use in human/veterinary medicine (Ament P. W et al., 2002), and agriculture (Heymann D.L. 2006) led the development of drug-resistant microorganisms and a fast increase in their frequency (Ferri M. et al., 2015).
A few years ago, The Infectious Disease Society of America (IDSA) confirmed that the resistant bacteria are and will be a risk to the United States and the rest of the world (Infectious Diseases Society of America. 2004). It is estimated that 30 percent of antibiotic prescriptions are for respiratory tract infections and more than one half of which are probably viral (Llor C. et al., 2014). The antimicrobial resistance is the cause of severe illness and their complications, of the longer hospital stays and often death of the sick (Kollef M. 2008; Paul M. et al., 2010; Livermore DM. 2012). In Europe occur annually 4 million infections by antibiotic-resistant germs that cause more than 37,000 deaths. This problem is due to the speed at which bacteria spread and change their genetic material. The antibiotic-resistance is the medical catastrophe of the third millennium (Holmes AH. et al., 2015), that threatens decades of scientific discoveries, and worse still, our health (Berkowitz FE. 1995). If antibiotic resistance continues to increase, it may become increasingly difficult to treat the diseases. Multifaceted interventions are needed to reduce the use of these drugs. In medicine is important the restriction of antibiotics only after medical examination or the laboratory tests, while in the farms there is the necessity to completely eliminate these drugs used to promote growth in animals or to prevent infectious diseases (Llor C. et al., 2014). Another way could be to find new antimicrobial agents to use against the resistant bacteria. Excellent candidates could be the antimicrobial peptides (Hancock R.E. et al., 2002) an ancient group of defense molecules produced in several plant, animal and invertebrate species. They attack the membrane bilayer of bacteria by carpet, toroidal-pore or barrel-stave mechanisms. The transmembrane pore formation is not the only mechanism that the antimicrobial peptides can use. They also inhibit nucleic-acid synthesis, alter cytoplasmic or inhibit enzymatic activity (Brogden KA. 2005). Thanks to the several mechanisms they can use against bacteria, the resistance towards these peptides is difficult to develop. Antimicrobial peptides have a broad range of activity against Gram-negative and Gram-positive bacteria, fungi, protozoa and yeast (Bal R. 2000). They are nontoxic to eukaryotic cells, this is due to the difference in composition of eukaryotic and prokaryotic membranes (Dathe M. et al., 1999). The eukaryotic membranes are rich in phospholipids zwitterionic such as phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, while in prokaryotic membranes there are phospholipids with negative charge (phosphatidylglycerol, phosphatidylserine and cardiolipin). The antimicrobial peptides are cationic and bind to membranes containing negative phospholipids due to electrostatic interaction. In last years, were discovered several proteins showing antibacterial activity not related to their primary function. These proteins seem to act as carrier of cryptic CAMPs which could be released by human or bacterial proteases. The cryptic antimicrobial peptides are often at the N-or C-terminus of the protein, and then, after their removal, can be easily activated. In this study, in collaboration with Dr. Notomista E. (Department of biology, University of Naples Federico II) was carried out a system of prediction to quickly identify cryptic antimicrobial peptides (Pane K. et al., 2016). This one correlates the structural characteristics of CAMPs with their antimicrobial properties (MIC). We have analyzed

the human proteins deposited into databases and between these the N-terminal isoform A3 (PAP3) of human pepsinogen was identified like potential CAMP. This antimicrobial peptide (47 aa) was expressed in *Escherichia coli* by fusing PAP3 to the C-terminus of Onconase (RNase from Rana pipiens) (Notomista E. et al., 1999). The Onconase (ONC) is a well suited partner for several reasons: it can be expressed at high levels as inclusion bodies (about 150 mg/L); no soluble onconase can be detected in the cultures thus minimizing the risk of toxic effects of the CAMPs; it is a small protein (104 aa) thus allowing higher yields of the peptides. Moreover ONC extracted from inclusion bodies is soluble at acidic pH (<4) but completely insoluble at pH 7. Therefore if the cleavage of the fusion protein is performed at acidic pH and successively the pH is increased up to 7, the ONC precipitates whereas the majority of the CAMPs will remain in solution thus allowing a simple purification of the peptide (Pane K. et al., 2016). The production of peptides as fusion proteins also poses the problem of separating the peptide from the carrier in order to obtain a pure peptide. For this it was necessary to interpose between the two parts a **GTGDP** linker sequence, as it is known in the literature the proteins containing DP sequences incubated at 60-80℃ in acidic environment can fragment, generating fragments that contain at the N-terminal a Proline residue. In addition, the fusion protein has a sequence of 6 Histidine residues located at the C-terminal end of onconase and the N-terminus of the peptide. In this way the protein was purified using affinity chromatography by chelation of metals (IMAC). This chromatographic system offers the possibility to purify proteins denatured in the presence of Guanidine-HCl 6 M, making this process independent from the value of the acidic pH needed to maintain in solution the protein onconase-PAP3 (Pane K. et al., 2016). PAP3 had a good antimicrobial activity against different pathogen bacteria. It was active on Gram-positive and Gram-negative bacteria, but it wasn't used in other experiments because it resulted toxic for human cells.

Another approach to the problem of antibiotic resistance is the use of bacteriophages (Bull J.J. et al., 2002). The idea of using bacteriophages to treat infections is old. In fact they were isolated for the first time by Hankins in 1896 (Topley WWC. et al., 1929). The phages were employed for a wide variety of pathogens like *Staphylococcus, Salmonella, klebsiella, Escherichia, Proteus* and *Pseudomonas* and the 92.4% of people infected from these bacteria were cured with phage therapy (Slopek S. et al., 1985). Recent researches have focused on the different mechanisms of phage delivery. The most popular is parenteral route but also local phage delivery (topical, otic, oral) and inhalation had successful. Local delivery of phages has proven very successful especially in former Soviet country (Kuter E. et al., 2005). The development of hydrogel impregnated of phages has received much attention, it has increased the success of wound healing. An example of commercial product is the Phagebio-derm which is active against *P. aeruginosa, S. aureus* and *Streptococcus spp*. (Markoishvili K. et al., 2002). The development of modern inhalation technologies has allowed also the use of bacteriophages to combat bacterial lung infections. The results suggested that phages can be nebulized and could be a potential approach for cystic fibrosis pulmonary infections. (Golshahi L. et al., 2011). The phage application as antimicrobials has focused on three family: *Myoviridae* with a big capsid (~150 nm) and contractile tail; *Siphoviridae* with a small capsid (~50-60 nm) and a long flexible, non-contractile tail; and *Podoviridae* with a small capsid (~50-60 nm) and a short tail (Kawa Z. et al., 2012). To enter in bacteria cells, bacteriophages must attach the specific receptors. This specificity (Ackermann

H-W. et al., 2007) of interaction between phage and receptor mostly influences the bacterial host range (Weinbauer M. G. 2004). Another crucial elements to establish if the phage is good as antimicrobial agent is the MOI (Ryan EM. et al., 2011). The Φ1 (isolated by *Salmonella rissen*) belonged to *Podoviridae* family had a good lytic activity host range 11/14 strains analyzed and the MOI 0,001. Furthermore the Φ1 genome consisted of 42,990 bp, 48.6% of G+C and 65 predicted coding sequences (CDSs). The Φ1 genome was accorded to the structure of T4 phage. This bacteriophage exhibited a genome functionally divided into three parts: preearly, early, and late regions. The preearly region includes a large terminally repeated sequence. The early genes are involved in phage metabolism, DNA replication, and lysis, also including a tRNA gene cluster. The late genes encode structural proteins for mature phage particles.

Phages are specific to the specie and not harm the natural flora when used as an antimicrobial to control a bacterial infection in humans (Salmond G.P.C. et al., 2015). Moreover the phages grow quickly and exponentially in this way it is possible to control bacterial infection with a single dose (Inal JM. 2003) and when the bacterial infection is eradicated even phages are eliminated. Nonetheless they are obligate parasites and therefore without their host have short half-life and aren't stable. Furthermore in contrast to antibiotics, no phage preparations for intracellular pathogens are available. In fact there are different works where avirulent bacteria are used to deliver the phages into infected cells (Broxmeyer L. et al., 2002). The Nanosciences and nanotechnologies have opened a new window on the development of nanomaterials used to administer drugs in cancer treatment, bacterial infections and wound healing (Kolanthai E. et al., 2017). A lot of nanoparticles such as carbon nanotubes, silica, metal oxide, grapheme and hydroxyapatite are frequently used for drug delivery (Chowdhury S. et al., 2016; Krishnamoorthy K. et al., 2014; Roveri N. et al., 2008). In this study the hydroxyapatite was chosen because it is not toxic even at high concentrations. This mineral (belonging to the apatite group) binds to several biological molecules (Lelli M. et al., 2016). The hydroxyapatite is similar to the constituents of human bones and it has good properties like biocompatibility, osteoconductivity, and degradability (Macchetta A. et al., 2009). For this reason he hydroxyapatite was used to solve the limits of bacteriophages. Studies conducted showed a greater stability of the complex compared to only phage: after several months stored at 4℃, the phage complexed with the hydroxyapatite maintains its title expressed in PFU/ml. Furthermore the hydroxyapatite has allowed the entry of the phages into eukaryotic cells. In this way is possible to use the phage therapy also against obligate *(Mycobacterium spp., Chlamydia spp., Rickettsia spp.)* or facultative *(Salmonella spp., Listeria spp., Brucella spp.)* intracellular bacteria (Silva M. 2012).

The phages could also be used in different steps of the food chain thanks to their specific antimicrobial activity (Colom J. et al., 2017). For example they could be administered with the food or water to farm animals to prevent or to eliminate intestinal pathogens. The acidic conditions of the stomach limit the phage viability, for this is necessary an delivery system to protect their orally administered (Ma Y. et al., 2008). This problem was successfully addressed by hydroxyapatite, in fact the complex (phage- hydroxyapatite) was more durable than the phage alone under acid condition, for this reason, it could be eligible to oppose gastro-intestinal pathogens. Salmonella outbreaks are common in chicken, cattle, pork and turkey due to poor hygienic conditions during slaughter and processing of meat (Doménech E. et al.,

2015). It is necessary that phages remain viable when used as biocontrol agents against contamination on work surface, in food packaging or directly added in food to eliminate the bacteria. (Cooper IR. 2016). In this study, the ground meat (previously infected with Salmonella) was treated with the complex (phage-hydroxyapatite) and phage. The contamination was significantly reduced from the complex, while the antimicrobial action of the phage alone was not significant. This is another proof that the hydroxyapatite protects the vitality of the phage.

6. CONCLUSIONS

After about 90 years from the discovery of antibiotics, we have reached a critical point in treating bacterial infection. Several powerful drugs that was highly efficient against bacteria are becoming useless. Bacteria resistant to multiple antibiotics threaten the global health, spoiling the benefits that were achieved with antibiotics. This problem reflects the inappropriate use of these drugs and the lack of new antimicrobial therapies able to keep up with bacterial evolution. Legislative interventions to modulate the antibiotic use are on the horizon, but new scientific approaches are necessary to resolve this pharmacological crisis (Nathan C. et al., 2005). In this Ph.D work were proposed two alternative to the antibiotics:

- Antimicrobial peptide;
- Bacteriophage.

The antimicrobial peptide used (PAP3) was identified by prediction system; in vitro it displays a satisfactory lytic activity against several pathogens, but it results toxic to eukaryotic cells, already at low concentrations. In this study, the PAP3 peptide was not further investigated.

Phages were successfully isolated from different food pathogens as *Salmonella typhimurium, Salmonella rissen, Listeria monocytogenes, and Escherichia coli*. The phage Φ1, isolated by *Salmonella rissen,* was chosen for further studies in view of its high lytic activity in vitro and large host range. Using electron microscopy and analyzing the DNA sequence, the phage Φ1 was classified as a member of the *Podoviridae* family. Its genome consists of three regions: pre-early, early, and late, thus reminding of the T4 phage.

This study has addressed the use of bacteriophages in humans, animals and in different steps of the food chain. The problem is that the phages are not stable, have a short half-life and cannot be used in case of intracellular infections because they are not able to enter the eukaryotic cells. In addition, the phages die at low pH of the stomach and when used during food processing and storage their viability can be compromised (Colom J. at al., 2017). These problems were solved successfully by complexing phages with hydroxyapatite. This complex is stable and able to enter eukaryotic cells and to remain active at pH 2. Strikingly, the complex killed the Salmonella artificially added to meat samples. Based on our results, we believe that the use of bacteriophages complexed with hydroxyapatite represents a promising biotechnological approach to treat / to prevent bacterial infections. Future challenges include the research of non-toxic antimicrobial peptides that can be used as antimicrobial agents alone or mixed with complex (phage-hydroxyapatite). Moreover it will be very interesting to understand the chemical interaction between phages and hydroxyapatite.

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LABORATORIES

Date (from-to)	Occupation or position held	Main activities	Name and andress of employer
01/04/2014-15/11/2014	PhD student	Biological characterization of peptide and phage	Immunology laboratory-University of Naples “Federico II”
16/11/2015-25/11/2015	Visiting PhD student	Stabilization of phage Φ1 on hydroxyapatite	Chemical Center Srl-Castello D’Argine (BO)
26/11/2015-18/02/2016	Visiting PhD student	Bacterial reduction assay on meat	Istituto zooprofilattico sperimentale del Mezzogiorno-(NA)
19/02/2016-22/07/2016	Visiting PhD student	Test on cells	De Montfort University-Leicester (UK)
23/07/2016-to date	PhD student	Data analyses and test on cells	Immunology laboratory-University of Naples “Federico II”

Dottorato in Scienze Biotecnologiche – XXIX ciclo

Università di Napoli Federico II

Dottoranda:	Flora Ianniello
Relatore:	Prof. Rosanna Capparelli
Coordinatore:	Prof. Giovanni Sannia

PUBBLICATIONS

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COMMUNICATIONS

Ianniello F. and Alfa H. Identification and characterization of antimicrobial agents to control bacterial infection. De Montfort University Annual Health and Life Science Postgraduate Research Conference held on Monday 27th June 2016.

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FEBS Letters

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Epistatic interaction between *MyD88* and *TIRAP* against *Helicobacter pylori*

Andrea Fulgione[1], Antonio Di Matteo[1], Felice Contaldi[1], Rosanna Manco[1], Flora Ianniello[1], Guido Incerti[1], Massimiliano De Seta[2], Nicolino Esposito[2], Antonio Crasto[1], Domenico Iannelli[1] and Rosanna Capparelli[1]

1 University of Naples 'Federico II', Italy
2 Fondazione Evangelica Betania, Naples, Italy

Correspondence
D. Iannelli, University of Naples 'Federico II', Via Università 100, 80055 Portici, Naples, Italy
Fax: +39 081 2531730
Tel: +39 081 2539277
E-mail: iannelli@unina.it

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The genes *MyD88* and *TIRAP* encode the adaptor proteins MyD88 and TIRAP. TIRAP plays the crucial role of activating the MyD88-dependent pathway, which in turn controls the immune response (innate and adaptive) to *Helicobacter pylori*. We looked for an association of *MyD88* and *TIRAP* with *H. pylori* infection. Cases and controls were genotyped at the polymorphic sites MyD88 rs6853 and TIRAP rs8177374 by real-time PCR. When the genes were analyzed separately, only TIRAP was associated with infection. When the genes were analyzed concurrently, certain combinations of *MyD88* and *TIRAP* protected the host against *H. pylori* colonization more efficiently than could be done by *TIRAP* alone.

Keywords: epistasis; *Helicobacter pylori*; *MyD88*; *TIRAP*

Helicobacter pylori colonizes about half the human population worldwide [1]. As far as it is known, *H. pylori* infection is limited to the human stomach [2], causing gastritis and more rarely peptic ulcers, gastric carcinoma or lymphoma. It is estimated that carriers have a 10–20% life-time risk of developing ulcers and < 1% risk of developing gastric carcinoma [1,2].

Studies conducted on myeloid differentiation factor 88-deficient ($MyD88^{-/-}$) mice have shown that the *MyD88* gene plays a major role in the immune response (innate and adaptive) to *H. pylori* [3]. MyD88 is an adaptor protein used by all Toll-like receptors (TLRs), except TLR3 [4]. Upon recognition of conserved pathogen-associated molecular patterns, TLRs signal the presence of the invading pathogen through the adaptor molecule MyD88, which in turn activates the immune response-inducing nuclear transcriptional factor NF-kB [5,6]. When infected *in vitro* with *H. pylori*, dendritic cells from *MyD88* wild-type ($MyD88^{+/+}$) mice express a large number of genes regulating the immune response (cytokine and chemokine production, antigen presentation, crosstalk between dendritic and T cells), cell differentiation, and cell cycle. Under the same conditions, dendritic cells from $MyD88^{-/-}$ mice do not express–or express at very low levels–the majority of these genes (especially of immune response genes) [3]. *MyD88* also directs T-cell differentiation. Infected *in vitro* with *H. pylori*, the dendritic cells from $MyD88^{+/+}$ mice display normal levels of IL-12 and Th1 phenotype, whereas the dendritic cells from $MyD88^{-/-}$ mice display reduced Th-1 phenotype and absence of IL-12, the cytokine driving T helper cells toward the Th-2 phenotype [3]. *MyD88* is also required for IL-1β, IL-6, IL-10 production [7]. An approach independent of the use of $MyD88^{-/-}$ mice confirms the importance of *MyD88* against *H. pylori* infection. *In vitro*, the microRNA miR-155 attenuates the expression of the MyD88 target protein and *H. pylori*-induced IL-8 [8]. As expected from *in vitro* studies, $MyD88^{-/-}$ mice infected with *H. pylori* show reduced gastric inflammation and increased

Abbreviations
MAL, MyD88 adaptor-like; ORs, odds ratios; TIRAP, TIR-domain-containing adaptor protein; TLRs, Toll-like receptors.

H. pylori colonization, compared with $MyD88^{+/+}$ mice [3]. Collectively, the above results demonstrate how the crucial aspects of the immune response against *H. pylori* are under the control of *MyD88*, *in vitro* and *in vivo*.

The TIR-domain-containing adaptor protein (TIRAP) (also called as MyD88 adaptor-like; MAL) is part of the TLR signaling pathway. Specifically, the activation of TLR7 and TLR9 is TIRAP-independent, while that of TLR2 and TLR4 is MyD88-dependent [9]. It has been suggested that the difference in TIRAP dependence might facilitate signaling specificity among individual TLRs [10]. The function of TIRAP is to bridge MyD88 to TLR2 or TLR4 [4] and thus activate the MyD88-dependent signaling pathway [9], which is directly involved in the recognition of *H. pylori*. TLR4 binds *H. pylori* [11], while TLR2 and TLR4 amplify iNOS induction [12], are expressed at high levels in the case of gastritis [13], and are associated with gastric cancer [14,15]. TLR2 displays also strong anti-inflammatory activity [16]. The rs10004195 within the TLR10/1/6 locus shows strong association with *H. pylori* seroprevalence and downregulates the TLR1 expression [16]. TLR1 and TLR2 form a heterodimer, predicted to bind *H. pylori* lipid A [16].

Here we show that, when analyzed individually, only *TIRAP* protects against infection. However, certain combinations of *MyD88* and *TIRAP* – when coinherited – strengthen protection against infection. The present data lend support to the approach based on the concurrent analysis of functionally related genes, which can reveal unexpected patterns of inheritance and favor the detection of disease-associated candidate genes with negligible effect, which escape detection when studied alone.

Materials and methods

Bacteria

Helicobacter pylori was grown as described [17]. Briefly, two specimens (from the antrum and corpus, respectively) of the same gastric biopsy were incubated in 1 mL Mueller-Hinton broth supplemented with 10% horse serum (Oxoid, Milan, Italy). Incubation (10 days) was under microaerophilic conditions (CampyGen, code CN0020C; Oxoid, Basingstoke, UK). At the end of the incubation time, cultures were streaked on *H. pylori* agar (code 413193; BioMérieux SA, Marcy l'Etoile, France) and incubated (10 days) under microaerophilic conditions.

Cases and controls

Cases and controls of the preliminary and confirmatory studies represent independent samples drawn from the same population. Criteria for definition of cases and controls were the same for both studies. The problem of hidden admixture was contained (as far as possible) by including in the study participants (cases and controls) who self-declared themselves as of Neapolitan ancestry (participants whose parents and grandparents were of Neapolitan ancestry) and lived in the same neighborhood (deduced from mail codes reported in the referral). They were referred to the Gastroenterology Unit of the local Villa Betania hospital (Naples) for gastric biopsy between January 2012 and December 2015. In the 8 weeks preceding gastric biopsy, participants were not treated with antibiotics, proton pump inhibitors or other drugs that could suppress bacterial growth. Patients with Barrett's esophagus or celiac disease –conditions negatively associated with *H. pylori* infection [18,19] – were excluded from the study. Cases (333 males and 490 females; mean age: 55 ± 10 years) were positive by the bacteriological (see above), hematoxylin-eosin [20], and PCR [21] tests for *H. pylori* infection. To exclude past infection, *H. pylori*-negative participants were further tested for the absence of the *H. pylori*-specific IgG antibody test (carried out according to the manufacturer's suggestions; abcam; code ab108736). Controls (338 males and 623 females; mean age: 54 ± 9) were participants negative in all tests listed above. The study was approved by the ethic committee of the Villa Betania hospital. Informed consent was obtained from all participants who participated in the study.

Genotyping

DNA was extracted from blood samples with the phenol-chloroform method [22]. Probes and TaqMan genotyping master mix were from Applied Biosystems (Life Technologies, Monza, Italy). Probes were specific for the polymorphic sites TIRAP rs8177374 and MyD88 rs6853. The PCR program included one step at 60 °C for 30 s, one at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and at 60 °C for 1 min and one cycle at 60 °C for 30 s. To validate genotype accuracy, the PCR products from 10% of the sample population plus all the participants carrying the rare genotypes (MyD88 GG or TIRAP TT) were sequenced. Two participants, who gave discordant results, were excluded.

RNA extraction and quantitative real-time PCR

Total RNA was extracted with the TRIzol reagent (Ambion by Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA) and reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Thermo Fisher Scientific Inc.). Real-time PCR was carried out with the Power Sybr Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific Inc.). Primer pairs were designed using the PRIMER 3 software (http://frodo.wi.mit.edu/primer3). Primer sequences are

reported in Table S1. Expression values were normalized against the levels of the human glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Statistical analysis was carried out with the GRAPHPAD PRISM version 5.03 (GraphPad, La Jolla, CA, USA).

Statistical analysis

Odds ratios (ORs) and 95% confidence intervals were calculated by Fisher's exact test using the statistical package GRAPHPAD PRISM version 5 (GraphPad). Logistic regression was calculated using the statistical package SPSS version 19 (IBM Corporation, Armonk, NY, USA).

Odds ratios were calculated from logistic regression model, which included *H. pylori* infection (as dependent binary variable); main and interactive effects of the MyD88 rs6853 and TIRAP rs8177374 polymorphic sites; age as potential confounders. Significance was tested by Wald's statistic, at $\alpha = 0.05$.

Protein network analysis (interactome)

Protein network analysis (interactome) was carried out using the STRING 10 database (http://string-db.org) [23]. The MyD88 and TIRAP proteins network is predicted based on neighborhood, gene fusion, co-occurrence, coexpression, text mining, and experiments available in the literature, or databases. The level of confidence and the maximum number of interactions between proteins were set at 0.7 and 5, respectively.

miRNA target prediction

The homologs of regulatory RNA motifs and elements binding the DNA sequence of the 3′UTR region of the *MyD88* common or rare alleles were identified using the REGRNA tool (http://regrna.mbc.nctu.edu.tw/). Of the regulatory RNA motifs supported by the REGRNA tool, we analyzed the most interesting for our study (miRNA target sites and motifs in mRNA 3′UTR). The level of stringency was that suggested by the tool.

Results

Validation of MyD88 and TIRAP as candidate genes

The selection of *MyD88* and *TIRAP* as candidate genes was a multistep process. The first step consisted in the review of *H. pylori* literature pertinent to our study (see previous section). Here, we describe the second step. The two adaptor proteins are part of the TLR network (or transcriptome) (Fig. 1), a term describing a complex of proteins cooperating to accomplish a biochemical function [24]. Within the TLR network, we identified the genes that act together against *H. pylori*: *MyD88* activating the downstream signaling pathway and *TIRAP* bridging MyD88 to TLR2 and TLR4 [4]. In the absence of TIRAP, the functions of TLR2 and TLR4 – two receptors playing a major role in the control of *H. pylori* infection [3,11,13] – are in fact compromised [10]. In cooperation with *TRAF6*, *TIRAP* also promotes the activation of NF-kB and consequently of the immune response [25]. This basic information is sufficient to illustrate the interdependence between *MyD88* and *TIRA*P (the two candidate genes) and *TLR2* and *TLR4* (the mediators of the immune response against *H. pylori*). Interactome studies provide valuable data on the protein interactions at the cell level. Yet they are prone to errors [26–28]. We therefore tried to support our candidate gene selection strategy using an independent approach, the crystal structure of the MyD88-IRAK4-IRAK2 complex analysis [29]. In this context, *MyD88*, *TIRAP,* and the other Toll/IL-1 receptor (TIR) domain-containing adaptors cooperate to create a platform favoring phosphorylation and NF-kB activation by the IL-1 receptor-associated kinases IRAK2 and IRAK4. Gene ontology analysis confirms the results described above (Table 1) and adds new ones (Table S2), such as the tendency of MyD88 and TIRAP proteins toward hetero-dimerization. This last finding, confirmed by different authors using different techniques [4,6,30], certainly adds weight to the hypothesis of a functional phenotypic interaction between the MyD88 and TIRAP molecules. On the basis of the above considerations, we selected *MyD88* and *TIRAP* as our candidate genes.

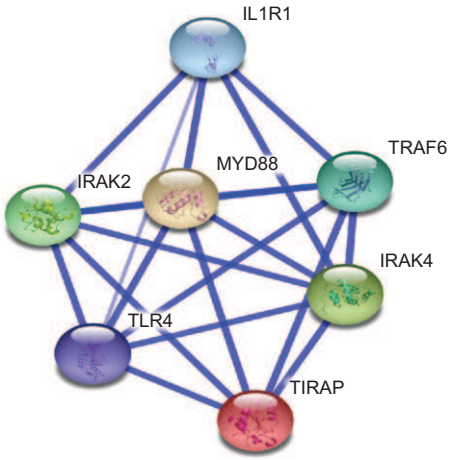

Fig. 1. Protein network analysis of human MyD88 and TIRAP proteins. Lines indicate interactions between proteins (or nodes). Nodes provide access to the three dimensional structure of proteins and additional information.

Table 1. Gene Ontology of terms common to MyD88 and TIRAP proteins.

Gene name	Gene name	Gene ontology terms	Category[a]	Description
MyD88	*TIRAP*	GO:0070935	P	3′-UTR-mediated mRNA stabilization
MyD88	*TIRAP*	GO:0032760	P	Positive regulation of tumor necrosis factor production
MyD88	*TIRAP*	GO:0038123	P	Toll-like receptor TLR1:TLR2 signaling pathway
MyD88	*TIRAP*	GO:0044130	P	Negative regulation of growth of symbiont in host
MyD88	*TIRAP*	GO:0005829	C	Cytosol
MyD88	*TIRAP*	GO:0043123	P	Positive regulation of I-kappaB kinase/NF-kappaB cascade
MyD88	*TIRAP*	GO:0046330	P	Positive regulation of JNK cascade
MyD88	*TIRAP*	GO:0051092	P	Positive regulation of NF-kappaB transcription factor activity
MyD88	*TIRAP*	GO:0002755	P	MyD88-dependent toll-like receptor signaling pathway
MyD88	*TIRAP*	GO:0050830	P	Defense response to Gram-positive bacterium
MyD88	*TIRAP*	GO:0038124	P	Toll-like receptor TLR6:TLR2 signaling pathway

[a] Gene Ontology associated categories: P (process); C (component).

Characterization of the SNPs selected for study

Of the 12 SNPs tested (MyD88: rs4988457; rs14814 9492; rs56269210; rs56271803; rs4988458; TIRAP: rs81 77400; rs8177350; rs8177351; rs8177369; rs8177373), only two (MyD88 rs6853 and TIRAP rs8177374) displayed a frequency of the rare allele > 0.05, a requisite for inclusion of SNPs in an association study [31]. The two SNPs are located on chromosomes 3 (location: 38184370; 3′UTR; NCBI) and 11 (location: 126162843; transition substitution; NCBI9), respectively. The MyD88 rs6853 polymorphism is reported to influence tuberculosis [32], the electroencephalogram pattern during anesthesia [33], and cognitive dysfunctions during chemotherapy [34] The TIRAP rs81773749 polymorphism is reported to confer resistance to malaria, tuberculosis, bacteremia, and invasive pneumococcal disease [35]. However, protection against tuberculosis has been confirmed by some, but not all, subsequent studies [32,36,37]. Recently, another TIRAP polymorphic site (rs793276) was associated with predisposition to gastric cancer [38].

Single locus analysis

The exploratory test hinted at the absence of the rare homozygous genotypes (*MyD88* GG and *TIRAP* TT) among cases and at a potential protective role of the *TIRAP* CT genotype against *H. pylori* infection (OR: 0.53; P: 9.1×10^{-5}; Table 2).

Association studies suffer from scarce reproducibility [39,40]. We therefore carried out a replication study, which supported the results from the preliminary study. It confirmed the protective role of the *TIRAP* CT genotype against *H. pylori* infection (OR: 0.56; P: 6×10^{-4}; Table 3) and the absence of the rare *MyD88* GG and *TIRAP* TT genotypes among cases (Tables 2 and 3). A recent study also describes the protective role played against *H. pylori* by the rare allele of the rs10004195 at the TLR1/10/6 locus [16].

Table 2. Association of *MyD88* rs6853 and *TIRAP* rs8177374 polymorphic sites with *Helicobacter pylori* infection. Preliminary study.

							Allelic frequency			
Genes	Status	Number of individuals in each genotype			Total	HWE (P)	Co[a]	Ra[b]	OR (CI)[C]	P-value
		AA	AG	GG						
MyD88	Cases	280	142	0	422	17.27 (3.2×10^{-5})	0.83	0.17	AG vs AA 1.19 (0.9–1.5)	0.22
	Controls	345	147	16	508	0.01 (0.92)	0.82	0.18		
		CC	CT	TT						
TIRAP	Cases	347	75	0	422	4.01 (0.045)	0.91	0.09	CT vs CC 0.53 (0.38–0.73)	9.1×10^{-5}
	Controls	348	141	5	494	5.16 (0.023)	0.85	0.15		

[a] Common alleles (*MyD88*: A; *TIRAP*: C).
[b] Rare alleles (*MyD88*: G; *TIRAP*: T).
[c] Confidence intervals.

Table 3. Association of *MyD88* rs6853 and *TIRAP* rs8177374 polymorphic sites with *Helicobacter pylori* infection. Replication study.

Genes	Status	Number of individuals in each genotype			Total	HWE (*P*)	Allelic frequency Co[a]	Allelic frequency Ra[b]	OR (CI)[c]	*P*-value
		AA	AG	GG						
MyD88	Cases	216	185	0	401	36.05 (0)	0.77	0.23	AG vs AA 1.16 (0.88–1.53)	0.29
	Controls	248	182	23	453	2.01 (0.15)	0.75	0.25		
		CC	CT	TT						
TIRAP	Cases	328	73	0	401	4.02 (0.044)	0.91	0.09	CT vs CC 0.56 (0.40–0.78)	6×10^{-4}
	Controls	320	126	7	453	1.88 (0.17)	0.85	0.15		

[a] Common alleles (*MyD88*: A; *TIRAP*: C).
[b] Rare alleles (*MyD88*: G; *TIRAP*: T).
[c] Confidence intervals.

Finally, the replication study confirmed lack of association between *MyD88* and the *H. pylori* infection (OR: 1.16; *P*: 0.29; Table 3).

MyD88-TIRAP epistasis

The adaptor protein TIRAP is required for the MyD88-dependent activation of TLR2 and TLR-4 [4,9], which in turn exert a crucial role in the immune response to *H. pylori* [3]. A tight cooperation between the TIRAP and MyD88 molecules is also inferred from their ability to form heterodimers [4,6,30]. We therefore looked for an epistatic interaction between the *MyD88* and *TIRAP*. *MyD88,* when alone, did not influence infection (Tables 4 and 5). Instead, in certain combinations with *TIRAP*, *MyD88* markedly influenced the risk of infection (Tables 4 and 5). In the preliminary and the replication studies, the intergenic interactions (*MyD88*/*TIRAP* AG/CT vs *MyD88*/*TIRAP* AA/CC) yielded the following ORs for infection: preliminary study: 0.16 (*P*: 5.3×10^{-6}); replication study: 0.12 (6.3×10^{-8}). In conclusion, *MyD88* displays its effect on *H. pylori* only when analyzed concurrently with *TIRAP*.

Table 4. Interaction between *MyD88* rs6853 and *TIRAP* rs8177374 polymorphic sites and *Helicobacter pylori* infection. Preliminary study.

Status	Interactions	OR[b]	*P*-value
	Allelic interactions		
Infection	*MyD88* (AG vs AA)	1.19	0.22
	TIRAP (CT vs CC)	0.53	9.1×10^{-5}
	Epistatic interactions		
	MyD88(AG) by *TIRAP*(CT)[a]	0.16	5.3×10^{-6}
	MyD88(AG) by *TIRAP*(CC)[a]	1.37	0.05
	MyD88(AA) by *TIRAP*(CT)[a]	0.75	0.12

[a] Reference genotype: *MyD88*(AA)/*TIRAP*(CC).
[b] Odds ratio.

Table 5. Interaction between *MyD88* rs6853 and *TIRAP* rs8177374 polymorphic sites and *Helicobacter pylori* infection. Replication study.

Status	Interactions	OR[b]	*P*-value
	Allelic interactions		
Infection	*MyD88* (AG vs AA)	1.16	0.29
	TIRAP (CT vs CC)	0.56	6×10^{-4}
	Epistatic interactions		
	MyD88(AG) by *TIRAP*(CT)[a]	0.12	6.3×10^{-8}
	MyD88(AG) by *TIRAP*(CC)[a]	1.72	8×10^{-4}
	MyD88(AA) by *TIRAP*(CT)[a]	1.27	0.25

[a] Reference genotype: *MyD88*(AA)/*TIRAP*(CC).
[b] Odds ratio.

After correction from age-confounding effect, double heterozygosity (*MyD88*/*TIRAP* AG/CT vs all the remaining allele combinations) still protected against infection (preliminary study: OR: 0.19; *P*: 2.4×10^{-4}; replication study: OR: 0.14; *P*: 2×10^{-6}). The single heterozygote *MyD88*/*TIRAP* AG/CC (vs all the remaining allele combinations) instead predisposed to infection (preliminary study: OR: 1.87; *P*: 6×10^{-5}; replication study: OR: 2.36; *P*: 8.3×10^{-8}), a result which clearly shows the protective role of the *TIRAP* CT genotype (Tables S3 and S4).

When the preliminary and replication studies were merged, the *MyD88* P value stands nonsignificant (0.22; 0.29; 0.08; Tables 2, 3, and 6); the *TIRAP* P value instead becomes significantly smaller (9.1×10^{-5}; 6×10^{-4}; 2×10^{-7}; Tables 2, 3, and 6); so do also the *P* values of the *MyD88-TIRAP* AG/CT and AG/CC epistatic interactions: (5.3×10^{-6}; 6.3×10^{-8}; 5.9×10^{-13}; Tables 4, 5, and 7) and (0.05; 8×10^{-4}; 10^{-4}; Tables 4, 5, and 7), respectively. In

Table 6. Association of *MyD88* rs6853 and *TIRAP* rs8177374 polymorphic sites with *Helicobacter pylori* infection. Combined study.

Genes	Status	Number of individuals in each genotype			Total	HWE (*P*)	Allelic frequency		OR (CI)[c]	*P*-value
							Co[a]	Ra[b]		
		AA	AG	GG						
MyD88	Cases	496	327	0	823	50.58 (0)	0.80	0.20	AG vs AA 1.18 (0.97–1.44)	0.083
	Controls	593	329	39	961	0.63 (0.42)	0.79	0.21		
		CC	CT	TT						
TIRAP	Cases	675	148	0	823	8.03 (4.6×10^{-3})	0.91	0.09	CT vs CC 0.54 (0.43–0.68)	2×10^{-7}
	Controls	668	267	12	947	6.7 (9.6×10^{-3})	0.85	0.15		

[a] Common alleles (*MyD88*: A; *TIRAP*: C).
[b] Rare alleles (*MyD88*: G; *TIRAP*: T).
[c] Confidence intervals.

Table 7. Interaction between *MyD88* rs6853 and *TIRAP* rs8177374 polymorphic sites and *Helicobacter pylori* infection. Combined study.

Status	Interactions	OR[b]	*P*-value
	Allelic interactions		
Infection	*MyD88* (AG vs AA)	1.18	0.08
	TIRAP (CT vs CC)	0.54	2×10^{-7}
	Epistatic interactions		
	MyD88(AG) by *TIRAP*(CT)[a]	0.14	5.9×10^{-13}
	MyD88(AG) by *TIRAP*(CC)[a]	1.53	10^{-4}
	MyD88(AA) by *TIRAP*(CT)[a]	0.94	0.73

[a] Reference genotype: *MyD88*(AA)/*TIRAP*(CC).
[b] Odds ratio.

conclusion, as expected, combining the two sets of data, makes the study more robust.

Different mRNA expression levels of the rs6853 alleles

The polymorphic sites located in the nonprotein coding region–such as the *MyD88* rs6853–often influence the phenotype by binding regulatory signals [41]. We therefore tested the mRNA expression levels of the three *MyD88* genotypes. We found that the GG genotype is significantly downregulated compared with the AA and AG genotypes (GG vs AA; $P < 10^{-4}$; GG vs AG; *P*: 3.6×10^{-3}) (Fig. 2). Using comparative genomics, we then attempted to identify the regulatory factors binding the rs6853 3′UTR. Of the nine factors examined (Table 8), two – the miR-526b-5p [42] and

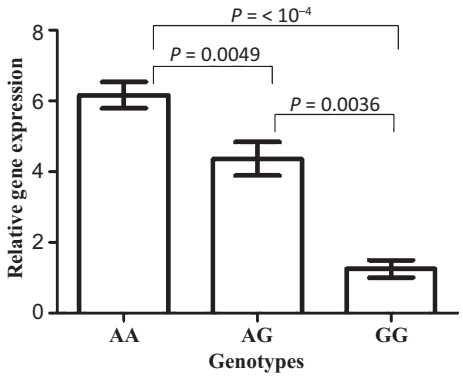

Fig. 2. Different mRNA expression levels of the *MyD88* rs6853 genotypes. Results are representative of three independent experiments. Each value represents the mean ± SD of determinations carried out in triplicate on 10 participants.

Table 8. Prediction of functional regulatory targets on the *MyD88* common allele.

Motif Type	Motif Name	Position	Length	Sequence
Transcriptional regulatory motif	TTF-1_(Nkx2-1)	1590–1601	12	atctcaagaggc
microRNA target sites	hsa-miR-18a-5p	1588–1607	20	ccatctcaagaggcatcttc
microRNA target sites	hsa-miR-18b-5p	1585–1607	23	aagccatctcaagaggcatcttc
microRNA target sites	hsa-miR-526b-5p	1580–1599	20	ttttaaagccatctcaagag
microRNA target sites	hsa-miR-1183	1585–1615	31	aagccatctcaagaggcatcttctacatgtt
microRNA target sites	hsa-miR-2682-3p	1583–1602	20	taaagccatctcaagaggca
microRNA target sites	hsa-miR-3668	1592–1613	22	ctcaagaggcatcttctacatg
microRNA target sites	hsa-miR-4657	1588–1613	26	ccatctcaagaggcatcttctacatg
microRNA target sites	hsa-miR-1273f	1573–1595	23	ctgggcattttaaagccatctca

Table 9. Prediction of functional regulatory targets on the MyD88 rare allele.

Motif Type	Motif Name	Position	Length	Sequence
microRNA target sites	hsa-miR-18a-5p	1588–1607	20	ccatctcgagaggcatcttc
microRNA target sites	hsa-miR-18b-5p	1585–1607	23	aagccatctcgagaggcatcttc
microRNA target sites	hsa-miR-1183	1585–1615	31	aagccatctcgagaggcatcttctacatgtt
microRNA target sites	hsa-miR-2682-3p	1583–1602	20	taaagccatctcgagaggca
microRNA target sites	hsa-miR-3668	1592–1613	22	ctcgagaggcatcttctacatg
microRNA target sites	hsa-miR-4657	1588–1613	26	ccatctcgagaggcatcttctacatg
microRNA target sites	hsa-miR-1273f	1573–1595	23	ctgggcattttaaagccatctcg

the transcriptional regulatory factor 1 (TTF-1) [43] – are predicted to bind the 3′UTR of the common, but not the rare rs6853 allele (Tables 8 and 9). Since the rs6853 common allele is not downregulated, miR-526b-5p (which downregulates the target mRNA expression) cannot explain our results (Fig. 2). With its property of modulating the expression of a large number of targets [43], TTF-1 remains the factor that potentially could explain the mechanism of the rare allele downregulation.

Inflammatory molecules expression levels among cases

The mRNA levels of the IL-6, COX-2, TNF-α, and IL-1β (collectively referred to as inflammatory molecules) varied among the *MyD88-TIRAP* genotypes. The inflammatory molecules were measured by real-time PCR and compared with the most frequent genotype among cases (AA/CC). The double heterozygous cases (AG/CT) displayed significantly ($P < 10^{-4}$) lower levels of the four inflammatory molecules (in particular of IL-6) compared with the double homozygous cases (AA/CC) (Fig. 3A). This result indicates: first, that the relative resistance of the AG/CT cases (OR: 0.12; P: 6.3×10^{-8}; Table 5) is associated with a marked low level of IL-6; second, that the *MyD88-TIRAP* interaction is evident at the statistical (Tables 4 and 5) as well as the phenotypic levels (Fig. 3A). Furthermore, single heterozygous cases (AA/CT and AG/CC) display significantly higher levels of IL-6 and COX-2 and – at the same time – significantly ($P < 10^{-4}$) lower levels of TNF-α and IL-1β compared with the double homozygous cases (Fig. 3B). These results are in line with the known role played by IL-6 in downregulating TNF-α and IL-1β [44,45] and upregulating COX-2 [46].

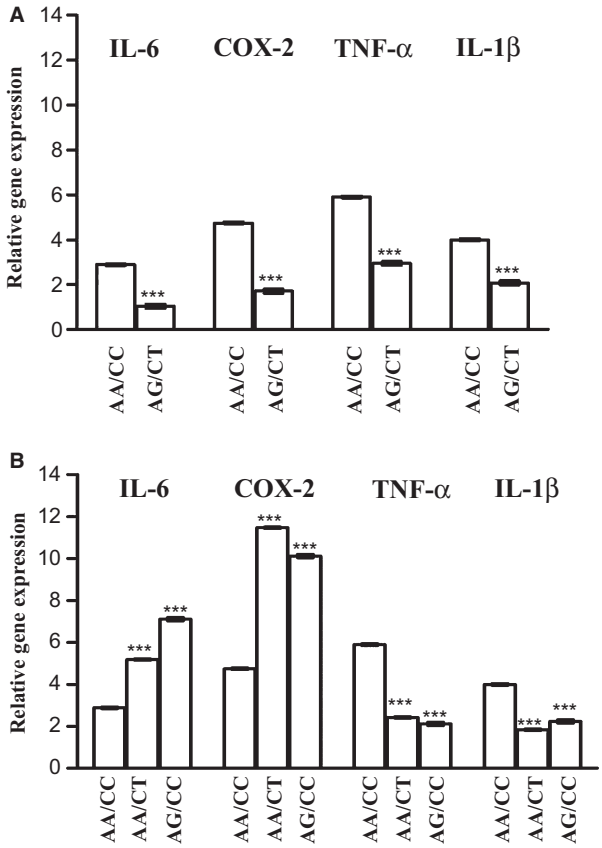

Fig. 3. Levels of IL-6, COX-2, TNF-α, and IL-1β mRNA in *MyD88-TIRAP* double (A) or single heterozygous (B) genotypes. Reference genotype: AA/CC. Results are representative of three independent experiments. Each value represents the mean ± SD of determinations carried out in triplicate on 10 participants AA/CC, AA/CT, AG/CC or on six participants AG/CT. ***P-value < 10^{-3}.

Discussion

When the genes *MyD88* and *TIRAP* were analyzed individually, the *MyD88* results failed to correlate with *H. pylori*. Instead, *TIRAP* – in the heterozygous form CT – confers resistance to infection (Tables 2 and 3). The MyD88 and TIRAP proteins are part of the TLR signaling pathway (Fig. 1). Their common function is to activate TLR2 and TLR4, two major receptors regulating innate and adaptive immune responses of the host against *H. pylori* [3,8,11,12]. In addition, the MyD88 and TIRAP proteins form heterodimers [4,6,30]. This evidence invited us to look for a potential epistatic

interaction between the corresponding *MyD88* and *TIRAP* genes, a contention supported by a previous study where the same genes acted epistatically against human pulmonary tuberculosis [32]. Indeed, both in the preliminary and the replication studies described here, certain combinations of *MyD88* and *TIRAP* protected the host against *H. pylori* colonization more efficiently than *TIRAP* alone could do, thus providing evidence of a nonadditive epistatic interaction occurring between the two genes (Tables 4 and 5). The epistatic interaction observed at the statistical level was also confirmed at the phenotypic level (Figs 3A and 3B).

The hypothesis that the epistasis described here could reflect a statistical artifact is unlikely. First, the property of the MyD88 and TIRAP adaptor proteins to form heterodimers has been demonstrated in independent studies and using different approaches, such as crystal structure analysis [4], coimmunoprecipitation and confocal microscopy [6]. Second, physical association between proteins coded by independent genes constitutes robust and unbiased evidence of epistasis [24]. Third, the same polymorphic sites acted epistatically also against *M. tuberculosis* [32]. In the near future, we will measure the epistatic effects potentially occurring between additional *MyD88* and *TIRAP* polymorphic sites, which we recently have identified. The study might lead to the discovery of new interactions influencing phenotypes correlated with *H. pylori* infection. *In vitro* studies on biopsy specimens might also lead to the discovery of possible perturbations of epistasis occurring in the presence of *H. pylori* and the potential effect of host genetic polymorphisms on this pathogen.

The finding that *MyD88* analyzed in isolation from *TIRAP* does not influence *H. pylori* introduces further interesting insights about this phenomenon. First, that epistasis can help to discover a new category of resistance genes, those with individual effects too weak to be detected when the gene is studied in isolation (this article). Second, that lack of replication of genetic association studies might in part reflect the presence of epistasis, a phenomenon that occurs more frequently than suspected [47]. Indeed, if the frequencies of two genes functionally linked by epistasis vary across populations, the frequencies of specific combinations also vary, contributing to the replication failure of association studies [48].

MyD88 and *TIRAP* confer resistance to *H. pylori* (this study) and *M. tuberculosis* [32]. Numerous diseases (infectious or autoimmune) are known to be influenced by the same genetic marker [35,49,50]. We add that one more potential benefit that might derive from studying genes coding for adaptor molecules is to find one therapy for several diseases. These molecules are in fact part of a hub integrating signals from a multitude of pathogens.

We feel cautiously optimistic about the reproducibility of the present study. Our optimism relies on the following facts. The preliminary and replication studies provide concurrent results; replication of results in an independent sample is the standard measure of replication [51]. The genes selected for study have strong biological plausibility and belong to the same biochemical pathway. Cases and controls have been accurately classified (cases being positive by the microbiological, PCR, and hematoxylin-eosin tests and controls negative by the same tests). The ever present hidden heterogeneity problem was contained (as far as possible) by including in the study participants (cases and controls), who self-declared themselves as of Neapolitan ancestry and lived in the same neighborhood.

In conclusion, the study shows first, that the two rare genotype (MyD88 GG and TIRAP TT) are not represented among cases (suggesting that they might confer total resistance to *H. pylori* infection). Second, double heterozygotes (MyD88-TIRAP AG/CT) display relative resistance to the pathogen (replication study; OR: 0.12; *P*: 6.3×10^{-8}; Table 5) and are characterized by lower levels of IL-6, COX-2, TNF-α, and IL-1β compared with double homozygotes (Fig. 3A). Third, double heterozygotes cases are characterized by a very low expression level of IL-6, a condition that by itself potentially could function as marker of relative resistance to *H. pylori*. However, we caution the reader that the clinical relevance of these findings needs to be first validated by independent studies on populations of different ethnicities. We finally caution that future replication of this study demands that *H. pylori* is detected using the bacteriological test (as we did). *Helicobacter pylori* diagnosis based on seroconversion could lead to noncomparable results.

Acknowledgement

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Author contributions

MDS, NE collected biopsy samples. AF, FI genotyped samples and carried out the microbiological tests. ADM, GI carried out the statistical analysis of data. FC carried out the bioinformatic work. RM carried out the real-time PCR experiments. AC contributed to the interpretation of experiments. DI, RC wrote the

manuscript. All the authors participated to the collective interpretation of the experiments.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Primer sequences.
Table S2. Gene Ontology of the MyD88 and TIRAP proteins.
Table S3. Preliminary test: age-confounding effect assessed by the weighted Mantel–Haenszel test.
Table S4. Replication test: age-confounding effect assessed by the weighted Mantel–Haenszel test.

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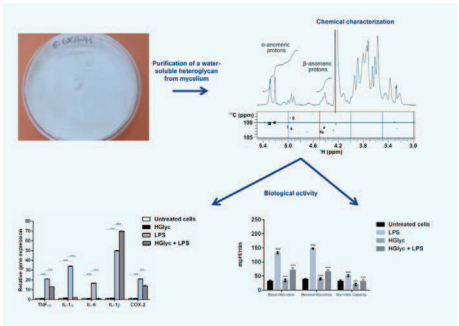
Structural data and immunomodulatory properties of a water-soluble heteroglycan extracted from the mycelium of an Italian isolate of *Ganoderma lucidum*

Raffaele Carrieri[a], Rosanna Manco[b], Daniela Sapio[b], Marco Iannaccone[b], Andrea Fulgione[b], Marina Papaianni[b], Bruna de Falco[b], Laura Grauso[c], Paola Tarantino[a], Flora Ianniello[b], Virginia Lanzotti[b], Ernesto Lahoz[a] and Rosanna Capparelli[b]

[a]Dipartimento di Colture Industriali, Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Caserta, Italy; [b]Dipartimento di Agraria, University of Naples Federico II, Portici, Napoli, Italy; [c]Stazione Zoologica "Anton Dohrn", Villa Comunale, Napoli, Italy

ABSTRACT

Mushrooms produce a wide range of bioactive polysaccharides, different from each other in chemical structure and biological effects. In the last years, the idea to develop functional foods or drugs containing fungal polysaccharides is attracting great attention. Fruiting bodies of Basidiomycetes *Ganoderma lucidum* are commonly used in Oriental medicine to treat several disorders. *G. lucidum* polysaccharides – mainly β-glucans and heteroglycans – have numerous biological properties such as antitumour and immunomodulatory activities. This report shows, by gene expression analyses and bioenergetic assays, immunomodulatory properties and capacity to improve glucose metabolism of a water-soluble heteroglycan extracted from mycelium of an Italian isolate of *G. lucidum*. The findings suggest the use of the heteroglycan as probiotic or ingredient in functional foods, being easy to produce and disperse in a food matrix thanks to its water-solubility. Heteroglycan could exert protective effects in pro-inflammatory conditions and benefits for people characterised by suppressed immune response.



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CONTACT Rosanna Capparelli capparel@unina.it
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1. Introduction

Fungi play an important role in several aspects of human life, in particular as source of therapeutics and food. Many fungi naturally produce antibiotics, which are able to inhibit the growth of the competing micro-organisms. The edible fungi (mushrooms) are often favoured in the human diet because they are poor in fat and rich in proteins, minerals and fibres (Manzi et al. 1999). Fungal cell walls are rich in bioactive polysaccharides (glycans), which are different from each other in chemical structure and biological activity (Herrera 2012). Glucans are the most abundant (and most studied as well) fungal glycans and they have only D-glucose as structural component: it is possible to distinguish linear or branched α-, β- as well as mixed α,β-glucans (Synytsya & Novak 2014). β-glucans of several fungi show numerous biological properties, including antitumour, antimicrobial, antidiabetic, antihypercholesterolemic and immunomodulating activities (Wasser 2011; Chang & Wasser 2012). Krestin – a heteroglycan isolated from *Polysticus versicolor* and consisted of glucose and other simple sugars – has antitumour, antihepatitis and antihyperlipidemic properties. Pachymaran – a heteroglycan purified from *Poria cocos* and consisted of glucose, galactose and mannose units – has antitumour activity and is used to treat insomnia and schizophrenia. *Grifola frondosa* heteroglycans – consisted of glucose, xylose, fucose, galactose and mannose units – show anticancer and immunomodulatory properties (Zhou et al. 2014).

Biological properties of fungal glycans vary depending on their water solubility, chemical composition and conformational structure (Polishchuk & Kovalenko 2009), but the basic requirements for biological activity are not still completely clear (Brown & Gordon 2003). Eight fungal glycans-based drugs are marketed in China, each of them displays unique polysaccharide composition and biological effects (Zhou et al. 2014). In general, water-soluble glycans are considered pharmacologically more active (Hu et al. 2013). Moreover, isolation methods may influence the features of the glycans and differences can be observed among compounds differentially isolated from the same source (Volman et al. 2008).

Ganoderma lucidum (Curtis) P. Karst is a lignocellulose-degrading mushroom of the Polyporaceae family. Over the past centuries, its fruiting bodies have been widely used in the Chinese and Japanese traditional medicine for the treatment of several disorders (such as gastritis, diabetes, hypercholesterolemia, hepatitis and cardiovascular problems). Modern research – by confirming the antitumour, antihypertensive, antidiabetic and immunomodulatory properties of *G. lucidum* extracts (Boh et al. 2007; Rex 2014) – has sensibly contributed to the success of this mushroom as nutraceutical so that the annual sale of *G. lucidum*-derived products is estimated about 2.5 billion U.S. dollars (Li et al. 2013). The major bioactive compounds of *Ganoderma* species are polysaccharides (such as β-glucans, glycoproteins and heteroglycans) and triterpenoids (Nie et al. 2013; Ruan et al. 2014), both usually isolated from the fruiting bodies of Asian isolates. In particular, polysaccharides are known for their anticancer and antimicrobial effects and their capacity to enhance the host immune system (Lin 2005), but there are few available studies about polysaccharides obtained from mycelium of European isolates of *G. lucidum* (Boh et al. 2007).

This study investigated immunomodulatory properties and capacity to improve glucose metabolism of a water-soluble heteroglycan (HGlyc), extracted from the mycelium of an Italian isolate of *G. lucidum*. For this purpose, gene expression analyses and two different bioenergetic assays were carried out in a cell line model. The report also describes an alkaline method, that combines purity with high yield, for the extraction of HGlyc and the chemical characterisation by NMR spectroscopy.

2. Results and discussion

2.1. Extraction of HGlyc

Eight grams of dry material were obtained from 80 g of fresh mycelium. About 20 mg of a water-soluble heteroglycan (composed by units of several monosaccharides) were extracted from 2 g of lyophilised mycelium. Simplicity and high yield suggest the possibility to use the method for a large-scale production of HGlyc from the mycelium of *G. lucidum*.

2.2. Monosaccharide composition and ^{1}H NMR spectrum

Preliminary studies were performed to determine the structure of HGlyc. The monosaccharide composition was determined by complete hydrolysis of the glycan at 100°C in water/TFA, followed by $NaBH_4$ reduction and acetylation with acetic anhydride. GC–MS analysis of the resulting alditol acetates showed the glycan to be composed of glucose, mannose, fucose, xylose and galactose, in the molar ratios 58:16:14:7:5 (see Experimental Part for details).

The ^{1}H NMR spectrum of the glycan in D_2O was recorded to obtain information about the anomeric configurations of the sugars. Firstly, the anomeric protons were identified from their correlation with the relevant anomeric carbons using an HSQC 2D NMR (Figure S1). To make ^{1}H signals as sharp as possible, the spectrum was acquired at 70°C. Even so, all signals appeared as unresolved broad singlets, and proton-proton coupling constants (the most reliable parameter for determination of anomeric configurations) could not be measured. Therefore, the determination of anomeric configuration was based on chemical shifts of anomeric protons (typically δ 5.2–4.9 for α-sugars and δ 4.6–4.3 for β-sugars) (Synytsya & Novak 2014). The anomeric region of the spectrum of HGlyc was quite complex (Figure S1), with multiple signals of different intensity both in the region of α-sugars and β-sugars. Integration showed an approximate 3:1 ratio between the overall areas of the signals of α-anomeric protons and β-anomeric protons, showing that most sugars were in the α configuration.

A more detailed analysis of the structure of HGlyc is in progress and the results will be reported in the due course.

2.3. Cell viability and NO_2 measurements

At the concentrations of 200, 500 and 1000 µg mL^{-1}, HGlyc did not affect cell viability, while the positive control (THP-1 cells treated with 50% ethanol) showed a 70% reduction of cell viability. Compared to the cells treated only with 10 µg mL^{-1} LPS, HGlyc (at the concentration of 200 µg mL^{-1}) reduced NO_2 production by 67% (Figure S2).

2.4. Bioenergetic analyses

Basal and maximal glycolysis values and glycolytic capacity were calculated (Figure 1(a–b)). Upon glucose addition, the medium of LPS-treated cells showed a significant increase of acidification (422%), compared to untreated cells, indicating a higher rate of basal glycolysis. Instead, when cells were treated with HGlyc and then LPS, a reduction of basal glycolysis was observed (53%). Next, incubating cells with oligomycine, the property of HGlyc to curb the maximal glycolysis was measured. Again, the HGlyc significantly reduced the maximal

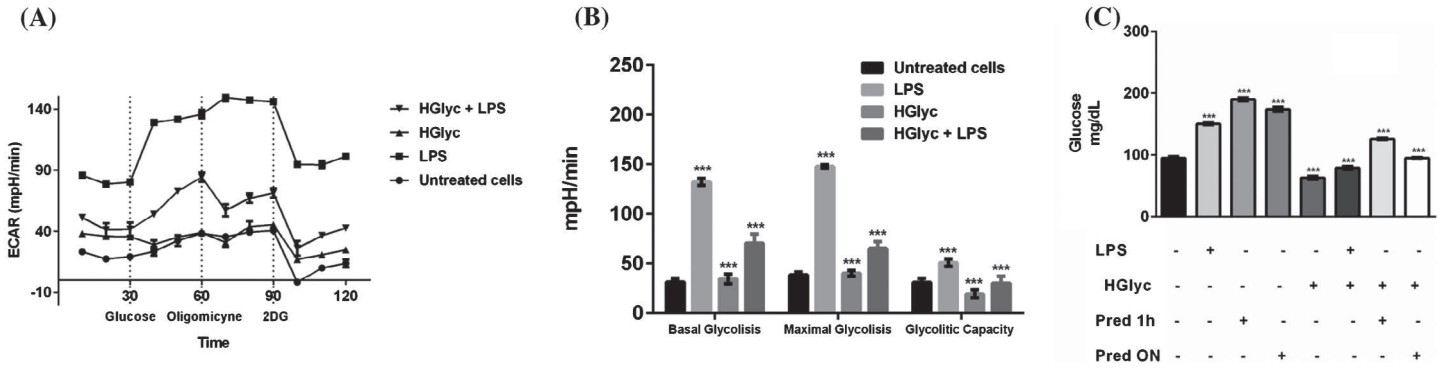

Figure 1. Bioenergetic assays. (a) ECAR parameters by extracellular flux technology. The values were measured, at baseline and after sequential addition of glucose, oligomycine and 2-DG, in: untreated THP-1 cells; cells treated 12 h with HGlyc 200 µg mL^{-1}; cells treated 1 h with LPS (10 µg mL^{-1}); cells treated with HGlyc and then LPS. Data are expressed as the rate of extracellular acidification in mpH/min. Data are shown as mean ± SE of triplicates for each measure. (b) Basal glycolysis, maximal glycolysis and glycolytic capacity. The parameters were measured in the following experimental conditions: untreated THP-1 cells; cells treated 12 h with HGlyc 200 µg mL^{-1}; cells treated 1 h with LPS (10 µg mL^{-1}); cells treated with HGlyc and then LPS. Data are expressed as mean ± SE of three measurements, each of them in triplicate. (c) Glucose-oxidase activity assay. Glucose amount secreted into the medium was measured in: THP-1 untreated cells; cells treated 1 h with LPS 10 µg mL^{-1}; cells incubated 1 h and 12 h (ON) with Prednisone 200 µg mL^{-1}; cells treated 12 h with HGlyc 200 µg mL^{-1}; cells treated with HGlyc and LPS; cells treated with HGlyc and Prednisone for 1 h or 12 h. Values represent the average determination ± SE for three experiments. Each experiment was carried out in triplicate.

glycolysis (44%). Finally, to measure the glycolytic capacity of the cells, 2-DG reagent was added. As expected, the glycolytic capacity was also significantly reduced (36%).

Glucose-oxidase activity, an index of glucose uptake in cells, was evaluated in eight experimental conditions (Figure 1(c)). Glucose level was markedly higher in cells treated with LPS or prednisone (1 h and 12 h), compared to untreated cells (58.5, 100 and 83%, respectively). LPS and prednisone are reported to determine an increase of glucose uptake, up-regulating the expression of glucose-transporter genes (Chung 2008; Hwang & Weiss 2014). Further, the property of HGlyc to curb the glucose uptake was measured: in cells treated with HGlyc and then with LPS or prednisone (1 h and 12 h), a reduction of glucose level (52, 66 and 95%, respectively) was registered. Cells treated only with HGlyc showed a glucose level lower (66%) than untreated cells.

Taken together, NO_2 measurements and ECAR values showed the capacity of HGlyc to reduce stress in inflammatory conditions induced by LPS in the model cell line. In our knowledge, ECAR analysis was used for the first time to investigate the effect of a fungal polysaccharide on cellular glucose metabolism. In addition, GOA assay indicated a hypoglycemic activity of HGlyc. Ability to reduce glucose levels in blood was already reported for polysaccharides from *G. lucidum* (He et al. 2006; Jia et al. 2009).

2.5. Gene expression

The expression levels of four important pro-inflammatory cytokines (TNF-α, IL-6, IL1-α and IL-1β) were measured. HGlyc was used at the optimal concentration of 200 μg mL^{-1}. The cells incubated with HGlyc and LPS displayed down-regulation of three cytokine genes: TNF-α by 28.5%; IL-6 and IL-1α by 94%, compared to the positive control (cells incubated only with LPS). On the contrary, IL-1β gene was up-regulated by 40%. The cytokines mRNA level was identical in cells treated with HGlyc and in untreated cells. The analysis was extended to the mediator of inflammation COX-2, that resulted significantly down-regulated (33% compared to positive control) (Figure 2). Down-regulation of pro-inflammatory molecules mediated

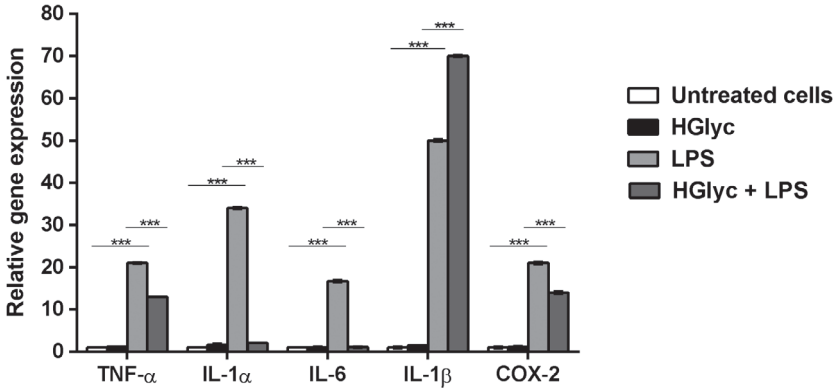

Figure 2. Expression levels of TNF-α, IL-6, IL1-α, IL-1β and COX-2 genes involved in immune response. The gene expression was evaluated in: untreated THP-1 cells; cells treated 12 h with HGlyc 200 μg mL^{-1}; cells treated 1 h with LPS (10 μg mL^{-1}); cells treated with HGlyc and then LPS. Values represent the average determination ± SE for three experiments, carried out in triplicate. A pool of THP-1 untreated cells was used as a calibrator for real time PCR experiments. *** = $p < 0.0001$. *P*-values express statistical significance for LPS-treated cells versus untreated cells, and for cells treated with HGlyc + LPS versus LPS-treated cells.

by *G. lucidum* polysaccharides was described in a previous report (Wang et al. 2014), in which water insoluble β-glucans extracted from fruit bodies reduced LPS-induced inflammation in mouse macrophages.

3. Conclusions

Different types of bioactive glycans – which vary in monosaccharide composition, glycosidic bonds, tertiary structure and molecular weight – have been purified from fruiting bodies of *G. lucidum* (Zhou et al. 2014). This study reports a method – easily reproducible – for the extraction of a water-soluble heteroglycan (HGlyc) from the mycelium of *G. lucidum*. HGlyc has glucose as major monosaccharide and it contains mannose, fucose, xylose and galactose. HGlyc exhibited the capacity to significantly reduce the inflammation induced by LPS in THP-1 cells in a dose-dependent manner. In cells incubated with HGlyc and then LPS, the expression levels of the mediators of inflammation TNF-α, IL-6, IL1-α and COX-2 were significantly down-regulated, compared to LPS-treated cells. On the contrary, HGlyc up-regulated the expression of IL-1β. This result is consistent with the property of fungal glucans to activate the transcription of this cytokine via the dectin-1-dependent pathway (Kankkunen et al. 2010). Bioenergetics assays and NO_2 measurements also confirmed the property of HGlyc to reduce cellular stress in inflammatory conditions, indicating at same time a hypoglycemic activity. HGlyc from *G. lucidum* are able to control excessive inflammation and to establish a homeostasis between pro- and anti-inflammatory responses, so they could be used as a probiotic or as ingredient in functional foods, with protective effects in pro-inflammatory conditions and benefits for people characterised by suppressed immune response.

In the last years, the idea to develop functional foods or drugs containing fungal polysaccharides is attracting great attention (Aida et al. 2009). Numerous health products based on *G. lucidum* are internationally marketed (Chang & Buswell 2008). Fruiting bodies are very rare in nature, so the amount of wild *G. lucidum* is not sufficient to meet the demands in international markets and its cultivation *in vitro* is essential. In the present work, a water-soluble heteroglycan was efficiently and economically isolated from the mycelium of *G. lucidum*, suggesting the possibility of a large scale production by submerged cultivations in bio-reactors. The benefits of liquid cultivations include: i) the ability to manipulate the medium to optimise mycelium growth; ii) reduced costs; iii) shorter cultivation time and lower risk of contamination, compared to the cultivation of fruiting bodies.

Furthermore, water solubility makes HGlyc easier to disperse in a food matrix and so more favourable for therapeutic applications, because they are easy to be absorbed by the organism and consequently more effective.

However, further studies are needed and these concepts should be confirmed in future trials *in vivo* using realistic food matrices.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Salmonella phage 29485, complete genome

GenBank: KY709687.1

FASTA Graphics

Go to:

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LOCUS       KY709687               51738 bp    DNA     linear   PHG 29-MAR-2017
DEFINITION  Salmonella phage 29485, complete genome.
ACCESSION   KY709687
VERSION     KY709687.1
KEYWORDS    .
SOURCE      Salmonella phage 29485
  ORGANISM  Salmonella phage 29485
            Viruses; dsDNA viruses, no RNA stage; Caudovirales; Podoviridae.
REFERENCE   1  (bases 1 to 51738)
  AUTHORS   Contaldi,F., Ianniello,F. and Capparelli,R.
  TITLE     Complete Genome Sequences of Podoviridae Bacteriophages Infecting
            Salmonella rissen
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 51738)
  AUTHORS   Contaldi,F., Ianniello,F. and Capparelli,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-MAR-2017) Department of Agricultural sciences,
            University of Naples Federico II, Via Universita 100, Portici,
            Napoli 80055, Italy
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