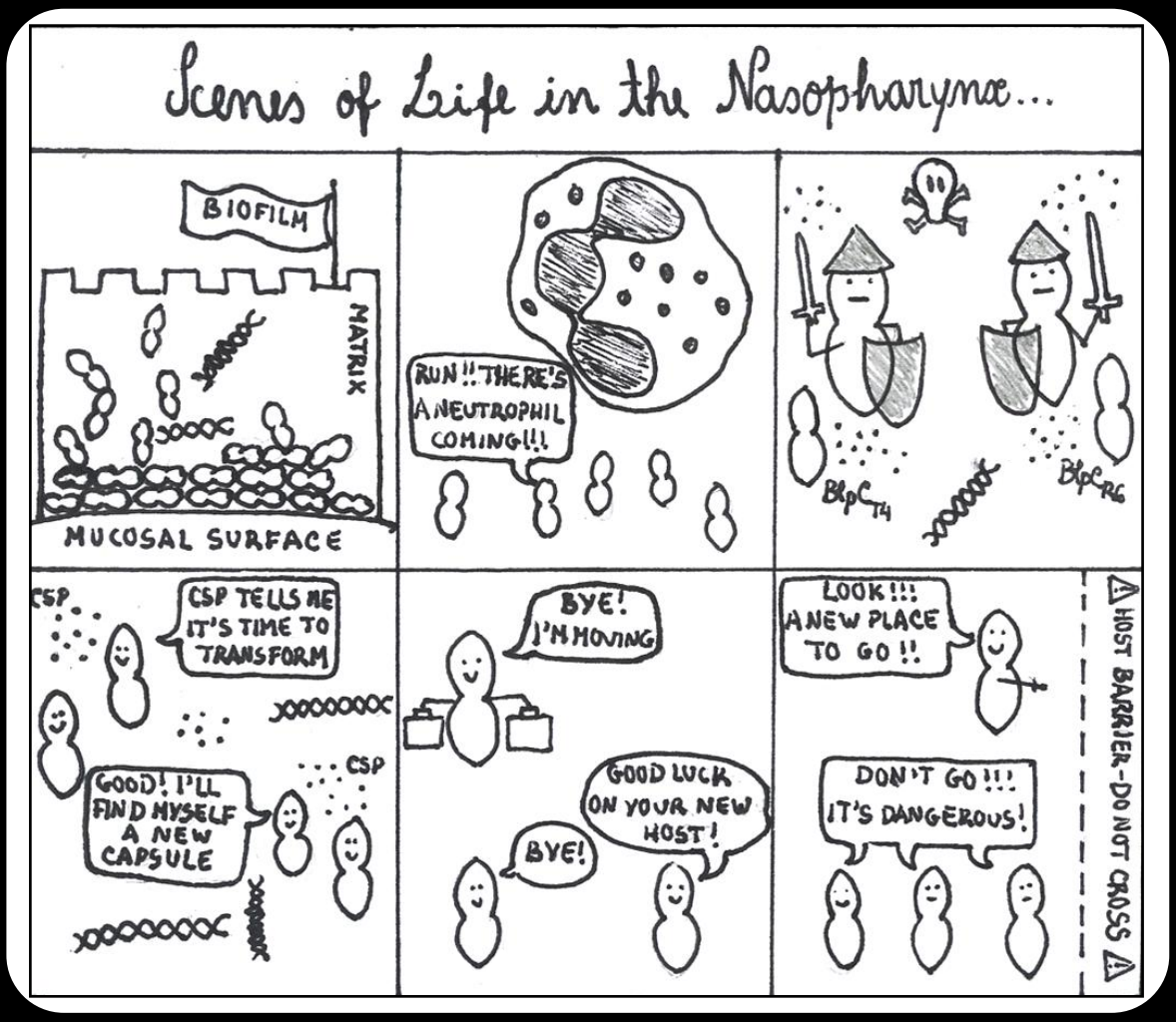
The role of co-colonization in pneumococcal ecology and evolution

Carina Valente



Dissertation presented to obtain the Ph.D degree in Biology | Molecular Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
December 2015

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“[…] searching for the causes of things is the best way to spend a life.”

Aristotle

in *Methaphysics*

Abstract

Streptococcus pneumoniae (or pneumococcus) is a frequent colonizer of the nasopharynx and an important cause of infectious diseases, with a high rate of morbidity and mortality worldwide, particularly among young children, the elderly and the immunocompromised. Despite this high burden of morbidity and mortality, invasive pneumococcal disease is incidental. Nasopharyngeal colonization is the preferred lifestyle for the pneumococcus, its prevalence being particularly high among young children

Colonization by more than one *S. pneumoniae* strain, or co-colonization, is frequent and is important for pneumococcal biology because it promotes intra-specific competition and evolution. Moreover, understanding co-colonization is critical to monitor the impact of pneumococcal conjugate vaccines (PCV) and the extent of serotype replacement by minor serotypes when vaccination targets the most abundant ones. Despite its relevance and the fact that it has been recognized for several decades, co-colonization remains poorly studied, mainly because high throughput methods of multi-type detection have been developed only recently.

In this thesis we conducted five studies that aimed at understanding the dynamics of pneumococcal co-colonization and how it is affected by pneumococcal conjugate vaccines and by specific properties and mechanisms intrinsic to *S. pneumoniae*.

In the first study we used a molecular-based strategy to detect co-colonization in nasopharyngeal samples from the pre- and vaccine eras, in order to evaluate the impact of the introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) on pneumococcal co-colonization in Portuguese children. We showed that co-colonization rates were significantly lower ($p=0.004$; Fisher's exact test) among fully vaccinated children (8.0%) than among children from the pre-PCV7 era (17.3%) or unvaccinated children of the PCV7 era (18.3%). We showed also that in PCV7

vaccinated children there was an asymmetric distribution of non-vaccine types (NVT) found in single and co-colonization events and we have attributed this asymmetry to differences in the competitive abilities of serotypes. We proposed that some NVTs prevalent in the PCV7 era are more competitive than others, impairing their co-existence in the same niche. The results obtained in this study may have important implications since a decrease in co-colonization events is expected to translate in decreased opportunities for horizontal gene transfer, hindering pneumococcal evolution events, and might represent a novel potential benefit of conjugate vaccines.

The replacement of PCV7 by PCV13 (13-valent PCV) has raised the question on whether this vaccine would have a similar effect on co-colonization. To address this question, in the second study we used a similar approach to the one used in the study conducted in the PCV7 era. We showed lower co-colonization prevalence in PCV13 fully vaccinated children, compared to children who had not received any PCV (20.6% vs. 29.3%), an observation that supports the results obtained in the first study. As before, we reported an asymmetric redistribution of NVTs in single and co-colonization events in the vaccinated group, which supported our hypothesis of different competitive abilities among NVT serotypes. Moreover, we showed that PCV13 serotypes are still highly prevalent in a population with no universal but very high vaccine coverage, mainly among non-vaccinated children. In vaccinated children PCV13 serotypes were found mainly as minor serotypes, while in the non-vaccinated group these serotypes were highly prevalent in single carriage events and were present in high relative abundance in most co-colonization events. Overall, the results obtained in this study regarding the impact of PCV13 on co-colonization corroborated the potential impact of PCVs on pneumococcal ecology by decreasing co-colonization events and, thus, decreasing opportunities for horizontal gene transfer.

In the third study we used knowledge previously obtained through the use of highly sensitive molecular methods of detection of co-colonization to quantify the bias

associated with the use of colony morphology for detection of co-colonization, a convenient approach that has been the basis of most reports on co-colonization in surveillance studies that do not systematically look for co-colonization. Comparison of the serotype distribution of co-colonized samples detected through both methodologies revealed that detection based on colony morphology resulted in a 20% overrepresentation of serotypes that display very distinctive colony morphologies, such as serotype 3 and non-encapsulated pneumococci, which did not reflect the real epidemiology of these serotypes on the pneumococcal population.

In the fourth and fifth studies we explored how co-colonization might be affected by specific properties and mechanisms intrinsic to *S. pneumoniae* biology.

In the fourth study we determined the impact of pherotype-mediated competition (or competence stimulating peptide, CSP) on pneumococcal co-colonization using a collection of co-colonized samples containing two strains of different serotypes. CSP assignment of all pneumococci revealed that in 52.5% of the samples both strains were of the same pherotype, while in 47.5% co-colonizing strains were of a different pherotype. Comparison of the observed proportions of concordant and discordant pherotypes with the ones estimated (53.8% and 46.2%, respectively), revealed no significant differences. The results obtained in this study supported the hypothesis that there is a limited role of pherotype-mediated competition on co-colonization.

In the fifth study we explored the impact of the *blp* (bacteriocin-like peptide) locus and bacteriocin secretion on co-colonization. We characterized the *blp* locus of a highly diverse collection of co-colonizing strains and determined their inhibitory activity through competition overlay assays. For comparison, we performed the same characterization in a collection of pneumococci isolated from single carriage events. We showed that co-colonizing pneumococci present high genetic diversity at the level of this locus and display several phenotypes of bacteriocin secretion and immunity. We

showed also that pneumococcal strains co-colonize individuals independently of the genetic content of their *blp*-locus and independently of their phenotypes of bacteriocin secretion, and that phenotypes of bacteriocin secretion are the same in strains isolated from single and co-colonization events. Overall, the results obtained in this study support the hypothesis that there is a limited role of the *blp*-locus and bacteriocin secretion on co-colonization.

In conclusion, the results obtained in this thesis improved the scarce knowledge on pneumococcal co-colonization through the use of innovative methodologies and by addressing important questions such as the impact of PCVs and bacterial competition mechanisms on this phenomenon and how they can influence pneumococcal ecology.

Resumo

A bactéria *Streptococcus pneumoniae* (pneumococo) coloniza assintomatica e frequentemente a nasofaringe e é também uma importante causa de doença infecciosa, com elevadas taxas de mortalidade e morbilidade, sobretudo em crianças, idosos e indivíduos imuno-comprometidos. A colonização da nasofaringe é, no entanto, preferencial no ciclo de vida do pneumococo, sendo particularmente elevada em crianças até aos seis anos.

A colonização da nasofaringe por mais do que uma estirpe de *S. pneumoniae*, ou co-colonização, é frequente. O estudo da co-colonização é importante dada a sua relevância para a biologia do pneumococo, por promover interacções intra-específicas e, consequentemente, a evolução da espécie. Além disso, compreender a co-colonização é fundamental para a monitorização do impacto das vacinas pneumocócicas conjugadas (PCV) e do grau de substituição de serótipos por serótipos minoritários, num cenário em que a vacina é dirigida contra os serótipos mais abundantes. Apesar de a co-colonização ser reconhecidamente importante e um fenómeno identificado há já várias décadas, continua pouco estudada, sobretudo devido ao facto de apenas recentemente terem sido desenvolvidos métodos adequados à sua detecção.

No âmbito desta tese foram realizados cinco estudos com o objectivo de entender a dinâmica da co-colonização por pneumococos e qual o impacto das PCVs e de certas propriedades e mecanismos intrínsecos à biologia desta bactéria neste fenómeno.

Com o objectivo de avaliar o impacto da introdução da vacina pneumocócica conjugada sete-valente (PCV7) na co-colonização por pneumococos em crianças portuguesas, realizámos o primeiro estudo, recorrendo a métodos moleculares para detectar co-colonização em amostras da nasofaringe de crianças obtidas antes e após a introdução da PCV7. Os resultados indicaram que crianças vacinadas com PCV7

completa apresentavam uma prevalência de co-colonização significativamente inferior (8.0%) à de crianças não vacinadas no mesmo período (18.3%) ou no período pré-vacinal (17.3%). Nas crianças vacinadas observou-se uma distribuição assimétrica dos serótipos não vacinais (NVT) nos eventos de co-colonização e colonização simples (*i.e.,* colonização por uma só estirpe), assimetria essa que atribuímos a diferenças nas capacidades competitivas dos serótipos. Esta observação levou à proposta de que alguns NVT prevalentes na era vacinal são muito competitivos, impedindo a sua co-existência com outros serótipos no mesmo nicho. Os resultados deste estudo poderão ter implicações relevantes dado que uma diminuição nos eventos de co-colonização poderá traduzir-se numa diminuição das oportunidades para a ocorrência de transferência horizontal de genes e, consequentemente, dos eventos de evolução, representando potencialmente um novo benefício das vacinas conjugadas.

A substituição da PCV7 pela PCV13 (PCV treze-valente) levou-nos a questionar se esta vacina teria um efeito semelhante ao da PCV7 na co-colonização. Deste modo, no segundo estudo adoptámos uma abordagem semelhante à do estudo da era da PCV7. Demonstrámos que a prevalência de co-colonização em crianças com vacinação completa (20.6%) era inferior à de crianças não vacinadas do mesmo período (29.3%). Em concordância com o estudo anterior, observámos uma distribuição assimétrica de NVT em eventos de co-colonização e colonização simples, facto que suporta a hipótese previamente proposta de diferenças nas capacidades competitivas dos serótipos. Demonstrámos ainda que os serótipos incluídos na PCV13 são ainda prevalentes na população. Nas crianças vacinadas os serótipos incluídos na PCV13 foram encontrados como um serótipo minoritário na maioria das ocorrências, enquanto que no grupo de não-vacinados estes serótipos eram muito prevalentes em eventos de colonização simples ou como serótipo maioritário em eventos de co-colonização. Os resultados apresentados neste estudo relativos ao impacto da PCV13 na co-colonização suportam a hipótese do que as PCVs podem alterar a ecologia do

pneumococo pela diminuição dos eventos de co-colonização e das oportunidades para transferência horizontal de genes.

No terceiro estudo usámos informação obtida previamente através do uso de métodos moleculares na detecção de co-colonização para quantificar o viés associado à detecção de co-colonização com base na morfologia das colónias de pneumococos. Dada a sua conveniência, esta abordagem baseada na morfologia constitui a base da maioria dos dados reportados na literatura relativos à prevalência de co-colonização. A comparação da distribuição de serótipos em amostras co-colonizadas com detecção efectuada através de ambas as metodologias demonstrou que a detecção com base na morfologia das colónias aumenta em 20% a prevalência de serótipos que apresentam morfologias mais facilmente distinguíveis, como é o caso do serótipo 3 e dos pneumococos não-encapsulados, podendo alterar artificialmente a prevalência estimada destes serótipos na população.

No quarto e quinto estudos, explorámos o impacto de propriedades e mecanismos intrínsecos à biologia do pneumococo na co-colonização.

No quarto estudo avaliámos o impacto da competição mediada pelo ferótipo (ou péptido indutor da competência) na co-colonização, recorrendo a uma colecção de amostras co-colonizadas por duas estirpes de pneumococos. A determinação do ferótipo para todas as estirpes revelou que 52.5% das amostras continham estirpes com o mesmo ferótipo, enquanto que nas restantes as estirpes eram de ferótipos diferentes. A comparação das proporções de amostras com ferótipos concordantes (52.5%) e discordantes (47.5%) com as proporções estimadas (53.8% e 46.2%, respectivamente) não revelou diferenças significativas. Estes resultados sugerem que a competição mediada pelo ferótipo tem um impacto limitado na co-colonização.

No quinto estudo explorámos o impacto do locus *blp* (*bacteriocin-like peptide*) e da secreção de bacteriocinas, *i.e.*, produção de péptidos antimicrobianos pelas bactérias,

na co-colonização. Caracterizámos o locus de uma colecção muito diversa de pneumococos identificados em eventos de co-colonização e determinámos a sua capacidade inibitória através de ensaios de competição em meio sólido. Utilizámos como controlo uma colecção de pneumococos isolados de eventos de colonização simples. Demonstrámos que os pneumococos a co-colonizar apresentam uma elevada diversidade genética ao nível do locus *blp* e exibem diferentes fenótipos de secreção de bacteriocinas e de imunidade. Adicionalmente, reportámos que os pneumococos co-colonizam um hospedeiro independentemente dos fenótipos de secreção de bacteriocinas e que os mesmos não são diferentes em estirpes de co-colonização e de colonização simples. Os resultados obtidos neste estudo sugerem que o impacto do locus *blp* e da secreção de bacteriocinas na co-colonização é limitado.

Em suma, os resultados apresentados nesta tese aumentaram o conhecimento sobre a co-colonização por pneumococos através do uso de metodologias inovadoras e da resposta a questões importantes, tais como o impacto das PCV e dos mecanismos de competição neste fenómeno, e a forma como os mesmos podem influenciar a ecologia do pneumococo.

Thesis outline

The purpose of this thesis was to gain insights on the dynamics of pneumococcal co-colonization and how it is affected by pneumococcal conjugate vaccines and by specific properties and mechanisms intrinsic to *S. pneumoniae* biology.

Chapter I is a general introduction where important aspects of *S. pneumoniae* epidemiology and biology, relevant for the scope of the thesis, are presented. Among these, are pneumococcal epidemiology, effects of anti-pneumococcal vaccination, importance of co-colonization, methods for detection of co-colonization, and bacterial properties and competition mechanisms that might impact on co-colonization.

Chapter II describes the assessment of co-colonization in a collection of nasopharyngeal samples obtained from vaccinated and non-vaccinated children, encompassing the availability of PCV7 in Portugal, aiming at determining the impact of this vaccine on co-colonization. The analysis includes the combination of two sensitive molecular methods for detection of co-colonization and comparison of co-colonization patterns among children sampled in the pre-PCV7 era, and vaccinated and non-vaccinated children sampled in the PCV7 era.

Chapter III describes a study conducted to evaluate the impact of PCV13 on co-colonization. Samples obtained from PCV13 vaccinated and non-vaccinated children were analyzed with a capsular microarray to detect co-colonization and the two groups were compared regarding co-colonization patterns.

Chapter IV describes a study conducted to assess the bias in the serotype distribution of pneumococci associated with co-colonization detection based on colony morphology. The strategy used was based on the comparison of the serotype

distribution of co-colonized samples detected by molecular methods and by colony morphology.

Chapter V describes the impact of pherotype-mediated fratricide on co-colonization. The analysis included pherotype assignment of a co-colonized collection of nasopharyngeal samples and estimation of the proportion of samples co-colonized with strains of the same or of a different pherotype for comparison with the observed proportions.

Chapter VI describes the impact of the *blp (*bacteriocin-like peptide*)* locus and bacteriocin secretion on pneumococcal co-colonization. The analyses included genetic characterization of the *blp* locus of co-colonizing strains and assessment of the phenotype of bacteriocin secretion through overlay assays. Comparison with a control collection of strains isolated from single carriage events was also performed.

Chapter VII presents general conclusions of the studies conducted in this thesis and enumerates several questions that remain unanswered and could be the focus of future research.

Chapters II, IV and V are reproductions of the following publications and can be read independently:

Chapter II - **Valente, C., J. Hinds, F. Pinto, S. D. Brugger, K. A. Gould, K. Mühlemann, H. de Lencastre, R. Sá-Leão**. 2012. Decrease in pneumococcal co-colonization following vaccination with the seven-valent pneumococcal conjugate vaccine. PLoS ONE; 7(1):e30235. doi: 10.1371/journal.pone.0030235.

Chapter IV - **Valente, C., H. de Lencastre, R. Sá-Leão.** 2013. Selection of distinctive colony morphologies for detection of multiple carriage of *Streptococcus pneumoniae*. Pediatr. Infect. Dis. J.; 32(6):703-4. doi: 10.1097/INF.0b013e31828692be.

Chapter V - **Valente, C., H. de Lencastre, R. Sá-Leão.** 2012. Pherotypes of co-colonizing pneumococci among Portuguese children. Microbe Drug Resist; 18(6):550-4. doi: 10.1089/mdr.2011.0228.

Chapter III has been submitted for publication and **Chapter VI** is nearly ready for submission.

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Chapter I

Introduction

Streptococcus pneumoniae, on the vanguard of scientific discoveries

Streptococcus pneumoniae, or pneumococcus, is an important human pathogen, continuing to kill thousands of people and being still "the captain of the men of death", as William Osler called it in 1918 (Osler, 1901).

The first reports about "elongated diplococci" in infected lungs are from Edwin Klebs and date back to 1875. It was only in 1881, however, that this bacterium was first isolated and its pathogenic potential was established, by both Louis Pasteur and George Sternberg (reviewed in (Watson *et al.*, 1993)).

Several designations were attributed to the pneumococcus along the years (reviewed in (Watson *et al.*, 1993)) taking into consideration the shape and type of disease caused by the bacteria but, in 1974, the name *Streptococcus pneumoniae* was adopted based on its morphology in chains when grown in liquid medium (Bergey, 1974).

Due to its important role as a human pathogen, *S. pneumoniae* is one of the most well studied bacteria. For this reason, it is not surprising that it has been center stage to important medical and scientific discoveries. In the field of medicine, the pneumococcus was used in the development of Gram's stain that allowed the distinction between Gram positive and Gram negative pathogenic bacteria (reviewed in (Watson *et al.*, 1993)), the discovery of the ability of polysaccharides to induce antibody production and their use as antigens in vaccines (Avery, 1917; Heidelberger & Avery, 1923), and the confirmation of the therapeutic efficacy of penicillin (Tillett *et al.*, 1944). In science, *S. pneumoniae* was the leading character in the discovery of the "Transforming Principle" and the mechanism of bacterial gene transfer (Griffith, 1928), in the identification of DNA as the genetic material (Avery *et al.*, 1944), in the discovery of the protective role of the capsule against host phagocytic cells (Felton *et al.*, 1955), and in the description of the first bacterial quorum-sensing mechanism (Tomasz, 1965),

all of which have contributed to even greater discoveries and, as of today, are still being used in the most various fields and applications.

Over 130 years have passed since the discovery of the pneumococcus and science and medicine are still far from winning the battle against this highly successful pathogen. Recent estimates on the number of serious cases of pneumococcal disease reach over 14 million, from which over 0.8 million cases result in the death of children below five years of age (O'Brien *et al.*, 2009) .

Streptococcus pneumoniae

S. pneumoniae is a Gram positive bacterium with the form of an elongated coccus (lancet-shaped). It varies between 0.5 and 1.5 µm in diameter and can be found isolated in single cells, in the form of diplococci, and in longer chains. Pneumococci are facultative anaerobic, display α-hemolytic activity when grown on blood-supplemented plates and are typically optochin susceptible and soluble in bile salts. It is a fastidious organism, its growth being favored in media containing blood, at 37ºC, and in a CO_2-supplemented atmosphere (CDC, 2012; Sneath, 1986).

The genome of *S. pneumoniae* is a covalently closed circular DNA molecule of ~2M base pairs, varying, in size and content, from strain to strain. It contains over 1500 genes that are essential for cell viability and a variable number of genes essential for virulence or to maintain a non-invasive phenotype (Bijlsma *et al.*, 2007; Hava & Camilli, 2002; Lanie *et al.*, 2007; Obert *et al.*, 2006; Orihuela *et al.*, 2004). *S. pneumoniae* possesses a highly plastic genome, with several recombination hotspots, which constitutes an evolutive advantage and makes it one of the most well adapted pathogens. The genomic variability of *S. pneumoniae* strains has been demonstrated (Chewapreecha *et al.*, 2014; Dagerhamn *et al.*, 2008; Hanage *et al.*, 2006; Hiller *et al.*,

2007; Obert *et al.*, 2006). In fact, analysis of 17 sequenced genomes showed that only 46% of the homologous gene clusters were common between strains (Hiller *et al.*, 2007).

Due to all the diversity in pneumococcal population and to the need to classify and cluster pneumococcal strains in the context of epidemiological studies, a gold-standard fingerprinting method, MLST (multi-locus sequence typing) is generally used as a typing method, in which a sequence type (ST) is assigned to a strain based on the combination of the allelic variants of seven housekeeping genes – *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt,* and *ddl* (Enright & Spratt, 1998) (http://www.mlst.net/).

One of most distinctive characteristics of the pneumococcus is its polysaccharide capsule, which led Avery to name it “the sugar-coated microbe” (Bardossi, 1988). This capsule has antigenic properties which can be used as an identification method, through the Quellung reaction, by the use of specific antisera (Sorensen, 1993). The capsule is the main pneumococcal virulence factor and has several distinct functions that will be discussed later in this chapter. Based on different antigenic properties conferred by biochemical and structural differences of the polysaccharide capsule, over 95 capsular types, or serotypes, have been described up to now (Calix & Nahm, 2010; Jin *et al.*, 2009; Oliver *et al.*, 2013; Park *et al.*, 2007; Park *et al.*, 2015).

Epidemiology of *S. pneumoniae*

Pneumococcal colonization

S. pneumoniae is a frequent inhabitant of the human nasopharynx, co-existing commensally with the human host. Colonization can occur soon after birth and remains high in the first three years of life, decreasing until the age of ten and remaining low during adulthood (Bogaert *et al.*, 2004a). Virtually every child is colonized by the

pneumococcus at least once in life and each serotype can colonize for several weeks or months, being then replaced by another serotype or reacquired (Gray *et al.*, 1980; Sá-Leão *et al.*, 2008).

Carriage is usually asymptomatic and its duration varies according to the serotype of the strain, the age of the child and the immunological status, as it allows the individual to acquire B cell mediated immunity against the carried serotype. A low mucosal immunity will result in persistent and repeated colonization events, while a stronger local response will accelerate clearance and prevent re-colonization (Bogaert *et al.*, 2004a; Weinberger *et al.*, 2008).

Asymptomatic carriers constitute a reservoir of pneumococci in the community and are important vehicles of transmission, which can occur via direct contact between individuals, by aerosols, or by contact with contaminated abiotic surfaces (Musher, 2003; Walsh & Camilli, 2011).

Risk factors for pneumococcal carriage include young age (up to 2 years of age), regular contact with young children, crowding (as occurs in day care centers, prisons, hospitals, and military camps), previous respiratory disease, both infectious and chronic, and cigarette smoking (Abdullahi *et al.*, 2012; Almeida *et al.*, 2014; Bogaert *et al.*, 2004a; Gray *et al.*, 1980; Labout *et al.*, 2008).

Several studies have been conducted worldwide on the subject of pneumococcal carriage (Adegbola *et al.*, 2014; Cohen *et al.*, 2012; Daana *et al.*, 2015; Vestrheim *et al.*, 2010) (Nunes *et al.*, submitted), most of them focused on young children attending day care centers, as this is the age group where colonization is more frequent and crowding is high in day care centers, favoring transmission (Bogaert *et al.*, 2004a; Sá-Leão *et al.*, 2008). These studies have shown that the carriage rates and the distribution of the carried serotypes vary across geographical locations, reaching values as low as 20% (Frederiksen & Henrichsen, 1988) and as high as 80%

(Vestrheim *et al.*, 2010). In Portugal, studies conducted in the Lisbon area report a pneumococcal carriage rate of ~64% in 2010 (Nunes *et al.*, submitted).

Pneumococcal disease

From the nasopharynx pneumococci can spread to other body sites to cause a wide range of diseases that vary in prevalence and severity. The most common forms of disease are mild infections such as acute otitis media (AOM) and sinusitis. The nasopharynx is connected to the middle ear via the Eustachian tube which, if clogged, can trap bacteria and cause a middle ear infection. Despite being a mild infection, AOM has a high socio-economic impact as it is the leading bacterial infection in children in high income countries and the primary reason for antibiotic prescription (Hau *et al.*, 2013). In fact, by the age of 3 years, over 80% of the children are estimated to have an AOM episode and over 40% to have recurrent episodes (Vergison *et al.*, 2010). Overall, the serotypes more frequently associated with AOM are serotype 3, 6A, 6B, 9V, 11A, 14, 19A, 19F and 23F, although this may vary with geographical location and vaccine coverage (discussed below in this chapter) (Rodgers *et al.*, 2009).

The pneumococcus can also be responsible for more severe infections, such as pneumonia (non-invasive disease), bacteremia and meningitis (invasive disease). Pneumonia is the non-invasive pneumococcal disease with highest burden in adults. It has high incidence rates and high mortality risk, especially in the elderly, and is the most common infectious source for invasive pneumococcal disease (Drijkoningen & Rohde, 2013).

The incidence of invasive pneumococcal disease (IPD) in any population depends on several factors, such as geographic location, season of the year, serotype prevalence, age, co-morbidities, and vaccine coverage (Drijkoningen & Rohde, 2013; Hausdorff *et al.*, 2005) (Figure 1). The highest reported incidence of IPD is in adults over 65 years of

age, children younger than 2 years of age, and those with certain underlying conditions that compromise the immune system, such as HIV infection and chronic diseases.

The World Health Organization estimated the occurrence of 14.5 million global cases of serious illness in children less than 5 years old (O'Brien *et al.*, 2009). According to the USA Active Bacterial Core surveillance (ABCs) database of the Emerging Infections Program Network, in 2013, the incidence of invasive pneumococcal disease in individuals ≥65 years of age was 30.5 cases per 100,000 population and, in infants ≤1 year, the incidence was 29.8 cases per 100,000 population (CDC).

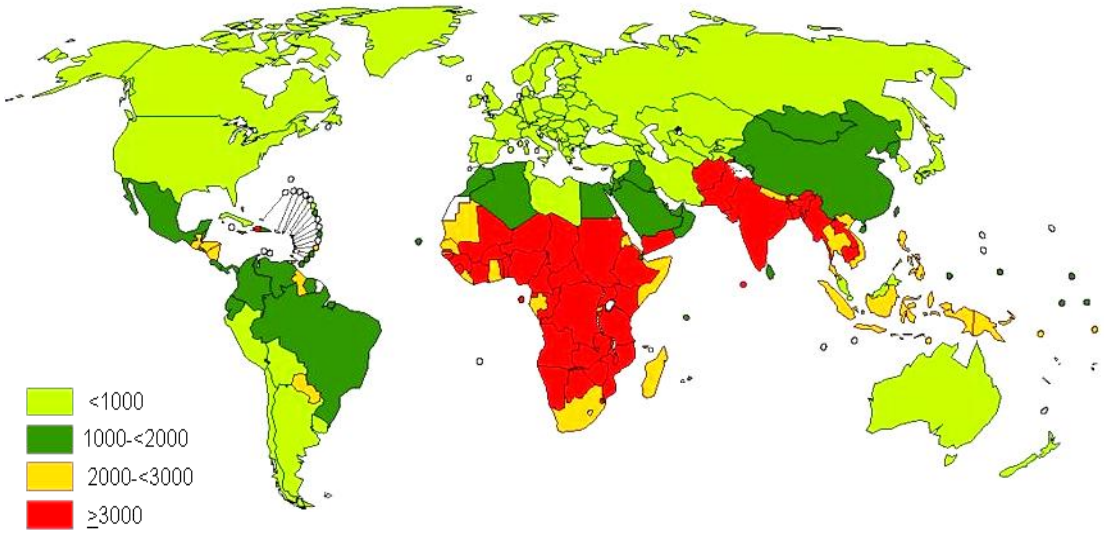

Figure 1. Incidence rate of invasive pneumococcal disease in children under 5 years old. (per 100,000 individuals). 2009. WHO website, adapted (http://www.who.int/).

The serotype prevalence in disease also varies with age, vaccine coverage and type of disease. Serotypes 4, 6B, 9V, 14, 18C, 19F and 23F were the most prevalent in pediatric invasive disease in the USA before the introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) (Hausdorff *et al.*, 2005), while serotype 19A emerged after PCV7 introduction (Richter *et al.*, 2009). Serotypes 1, 4 and 14 have been associated with bacteremia (Hausdorff *et al.*, 2000). Serotype 1 has also been associated with pneumonia, together with serotype 3 (Hausdorff *et al.*, 2005).

In Portugal, a study has shown that serotypes 1, 7F and 19A accounted for 61% of pediatric invasive pneumococcal disease cases between 2006 and 2008 (Aguiar *et al.*, 2010).

From the information provided above it is clear that, although there are over 95 serotypes described, only a few are responsible for causing disease. Several studies have aimed at establishing differences in invasive potential among serotypes (Brueggemann *et al.*, 2003; Brueggemann *et al.*, 2004; Greenberg *et al.*; Sá-Leão *et al.*, 2011; Sleeman *et al.*, 2006). These studies had different designs and were conducted in different geographical locations but the overall results were similar, as most reported a high invasive disease potential for serotypes 1, 3, 4, 5, 7F, 9V, 14, and 18C. The study conducted in Portugal identified the same serotypes as having a high invasive potential, among others, while serotypes 6A, 6B, 11A, 15B/C, 16F, 19F, and 23F, among others, to be mostly associated with colonization (Sá-Leão *et al.*, 2011). More importantly, this study has shown that different genetic lineages of the same serotype have different invasive potential, as is the case of serotypes 3, 6A, 6B, 19A, 19F, and 23F, among others.

Non-susceptibility to antimicrobial agents

Non-susceptibility of *S. pneumoniae* to commonly used antibiotics has been reported worldwide (CDC; ECDC, 2013) and has increased dramatically since the description of the first clinical isolate resistant to an antimicrobial agent (penicillin) in 1967 (Hansman *et al.*, 1971).

The distribution of non-susceptible isolates seems to be associated with the capsular type, the geographical area and antibiotic consumption levels of each area, and with

pneumococcal conjugate vaccine coverage (Dagan & Klugman, 2008; Goossens, 2009; Hausdorff *et al.*, 2005; Riedel *et al.*, 2007).

In general, high rates of non-susceptibility are observed in countries with high antibiotic consumption, such as Southern and Eastern European countries, whilst low non-susceptibility rates are observed in countries with low antibiotic consumption, such as Northern European countries (Goossens, 2009; Riedel *et al.*, 2007). Accordingly, strains isolated from carriage tend to be more frequently non-susceptible to antimicrobial agents than strains isolated from disease episodes (Hausdorff *et al.*, 2005).

The latest report from the European Center for Disease Control and Prevention (ECDC) showed that, in 2013, the percentage of isolates non-susceptible to penicillin causing invasive disease ranged between < 1.1% (the Netherlands) and 40.0% (Cyprus), while for macrolides non-susceptibility rates ranged between 1.5% (Latvia) and 38.1% (Romania) (ECDC, 2013). In the USA, in the same year, the percentages of penicillin and macrolide non-susceptible isolates were 4.7% and 28.5%, respectively (CDC).

In Portugal, data from carriage isolates from 2010 showed percentages of non-susceptibility to penicillin and macrolides of 19.6% and 25.2%, respectively. The same study showed that in that period, non-susceptibility to those antimicrobial agents was associated mainly with serotypes 6C, 15A, 19A, 19F, and non-encapsulated isolates (Nunes *et al.*, submitted).

A study focused on isolates causing invasive disease in Portugal, from 2006-2008, showed that the rates of non-susceptibility to penicillin and macrolides were 18.7% and 22.9%, respectively. This study has also shown that non-susceptibility to those

antimicrobial agents was associated mainly with serotypes 19A, 14, 19F, 6C and 23F (Aguiar *et al.*, 2010).

Anti-pneumococcal vaccination

Being *S. pneumoniae* "the captain of the men of death" (Osler, 1901), it is not surprising that several attempts have been made throughout history to prevent pneumococcal disease through vaccination.

Although not effective, the first attempt was the use of killed whole bacterial cells to immunize mineworkers, in 1911, to prevent death by pneumonia (Wright *et al.*, 1914). The demonstration of the efficacy of pneumococcal vaccines was a long process, mainly due to flawed study designs and serious adverse effects of the vaccines. Although some clinical trials were undertaken, the discovery of antibiotics and their efficacy against pneumococcal disease put this process on hold, until Robert Austrian proved the efficacy of pneumococcal polysaccharide vaccines and, as a consequence, the 14-valent polysaccharide vaccine was licensed (Austrian *et al.*, 1976). In 1983 this vaccine was extended to 23 serotypes and licensed, being still in the market with the commercial name of Pneumovax© 23 (PPV23). PPV23 is immunogenic for serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F when administered in adults, the elderly and children older than 2 years of age, as in younger children, who have an underdeveloped immune system, it fails to induce an adequate immune response (Bogaert *et al.*, 2004b; Douglas *et al.*, 1983).

In 2000, the first pneumococcal conjugate vaccine (PCV7) was licensed and introduced in the National Immunization Plan in the USA, with the commercial name of Prevnar©, and protecting against the seven serotypes more prevalent in pneumococcal disease in children under 6 years of age: 4, 6B, 9V, 14, 18C, 19F, and 23F (CDC, 2000). The polysaccharide of these serotypes was conjugated to a protein carrier, CRM_{197}, resulting in a better immunogenicity in young children.

Two additional conjugate vaccines have been developed in more recent years: a 10- and 13-valent vaccine, in 2009 and 2010, respectively (Synflorix© and Prevnar©13, respectively). Synflorix© (PCV10) includes the 7 serotypes present in PCV7 and 3 additional serotypes - 1, 5 and 7F – conjugated to *Haemophilus influenza* protein D as the carrier protein. Prevenar©13 (PCV13) replaced PCV7 in 2010 and includes six additional serotypes, conjugated to the same carrier protein, CRM_{197}: 1, 3, 5, 6A, 7F, and 19A.

In Portugal, PCV13 was introduced in the National Immunization Plan for children born after January 2015 on a scheme of two doses followed by a booster dose. Nevertheless, PCVs have been available in the market since June 2001 (PCV7), April 2009 (PCV10) and January 2010 (PCV13) and their use in young children has been recommended by the Pediatric Portuguese Society. Estimates on PCV7 coverage in 2009 reached 62%, while PCV10 coverage was estimated to be 13% in the same year (data from the National Statistics Institute (INE) and IMS).

Effects of pneumococcal vaccination

The introduction of PCV7 has led to several changes in pneumococcal epidemiology and population structure. These changes are more or less significant according to the vaccine coverage and geographic location and included: (i) decrease in IPD in vaccinated children, (ii) decrease in IPD in non-vaccinated children and in adults, as a reflex of the herd-immunity effect, (iii) increase in the incidence of IPD caused by non-vaccine serotypes, and (iv) replacement of vaccine types by non-vaccine types in carriage. The USA, a country with immediate massive PCV7 use, is a good example of all these changes. A 77% decrease in IPD cases in vaccinated children was reported four years after vaccine introduction (CDC, 2008) and later a 45% decrease in IPD in all age groups was observed, accompanied by a 94% reduction of vaccine types (Pilishvili *et al.*, 2010). Additionally, the incidence of invasive disease cases attributed

to non-vaccine types also increased in the first years of vaccination, particularly due to serotype 19A (Hicks *et al.*, 2007).

In Europe, the same effects were observed but with some variability among countries, depending on vaccine coverage. Decrease in IPD incidences varied between 28% and 68% and serotype replacement was also observed, with non-vaccine serotypes 1, 19A, 3, 6A, and 7F being the ones with higher incidence in IPD after widespread use of PCV7 (reviewed in (Isaacman *et al.*, 2010)).

In Portugal, the same scenario was observed. The incidence of IPD cases associated with PCV7 serotypes was reduced from 56% (before PCV7 introduction) to 17% by 2006-2008, in children aged up to 17 years of age (Aguiar *et al.*, 2010). Carriage of PCV7 serotypes also decreased, while the prevalence of non-PCV7 serotypes increased, maintaining the overall prevalence of carriage in the population (Sá-Leão *et al.*, 2009) (Nunes *et al.*, submitted).

Since serotypes included in PCV7 are frequently resistant to antimicrobial agents, introduction of PCV7 vaccination was predicted to have an impact on the levels of antimicrobial resistance worldwide (Dagan & Klugman, 2008; Kyaw *et al.*, 2006). This decrease was, in fact, observed following the first years of vaccination but was rapidly compensated by the emergence of drug-resistant non-PCV7 serotypes (Mera *et al.*, 2009). In Portugal, antimicrobial resistance rates were maintained, despite the widespread use of PCV7 (Simões *et al.*, 2011) (Nunes *et al.*, submitted).

PCV10 and PCV13 have similar effects on the population as the ones described for PCV7, with reports on the impact these vaccines becoming more frequent (Hammitt *et al.*, 2014; Jokinen *et al.*, 2015; Moore *et al.*, 2015; Waight *et al.*, 2015). In the USA a study reported a decrease of 93% in IPD cases attributable to PCV13-only serotypes, in children under 5 years old, and a decrease of 58-72% in adults, depending on age, for the same serotypes (Moore *et al.*, 2015). In the UK, the reduction of PCV13-only

serotypes IPD cases was of 69% for all ages (Waight *et al.*, 2015). However, both studies reported an increase in IPD cases associated with non-PCV13 serotypes, particularly in children under 5 years old, evidence of the serotype replacement expected to occur.

Despite the serotype replacement that has been observed and that is attributable to the fact that current vaccines only target a limited number of serotypes, the strategy of targeting the most prevalent serotypes in IPD has been highly successful. However, the adaptability of the pneumococcal population to these human interventions has been a driving force for the development of the ideal vaccine. This vaccine would be a conserved, universally present surface protein, capable of eliciting a good immune response in all age groups. No such protein has been described so far but several initiatives are ongoing, using surface proteins, combinations of proteins or an whole cell approach to find a vaccine that could ultimately overcome the limitations of currently used conjugate vaccines (reviewed in (Feldman & Anderson, 2014)).

Pneumococcal co-colonization

Biological significance

The preferential niche of the pneumococcus is the nasopharynx, a polymicrobial environment where the pneumococcus is forced to interact with other microorganisms of the same or different species. Simultaneous colonization by more than one pneumococcal strain, or co-colonization, is frequent and is of high clinical, epidemiological and ecological relevance (O'Brien & Nohynek, 2003; Shak *et al.*, 2013).

It has been shown that colonizing pneumococcal strains can interfere both negatively and positively with colonization by other strains (Dawid *et al.*, 2007; Lipsitch *et al.*,

2000; Marks *et al.*, 2012b). Marks *et al.* (Marks *et al.*, 2012b) have shown in a mouse model that strains with poor colonization efficiency have their efficiency improved when in co-colonization with a highly efficient strain that is already established. This improvement was independent of genetic exchange between strains. On the other hand, Lipsitch *et al*. (Lipsitch *et al.*, 2000) have shown, also in a mouse model, that carriage of an established strain can inhibit acquisition of a second strain in a strain- and density-dependent manner. The inhibition was observed through a reduced colonization probability and/or through reduced cell counts in the colonized animals. Additionally, some molecular mechanisms have been shown to contribute to inter-strain interactions, namely bacteriocin production and competence-mediated fratricide, which will be discussed later in this chapter (Dawid *et al.*, 2007; Guiral *et al.*, 2005).

Co-colonization is a requirement for horizontal gene transfer between pneumococci through homologous recombination, a mechanism important for evolution in this species (Coffey *et al.*, 1991; Feil *et al.*, 2000; Smith *et al.*, 1993; Spratt *et al.*, 2001). Several studies reported the occurrence of genetic exchange in biofilm models (Carrolo *et al.*, 2014; Marks *et al.*, 2012b; Wei & Havarstein, 2012) and also in *in vivo* models (Marks *et al.*, 2012b). Additionally, Hiller *et al.* (Hiller *et al.*, 2010) have shown the occurrence of genetic rearrangements *in vivo* that generated genetic diversity during a pediatric chronic infection. Several population genetic studies have also shown the occurrence of genetic recombination, including capsule switching events (Croucher *et al.*, 2011; Donkor *et al.*, 2011). Donkor *et al.* (Donkor *et al.*, 2011) have used an MLST approach to characterize pediatric colonization strains and they found evidence of extensive recombination among those strains, which they have related to the high prevalence of co-colonization in the population.

Besides the evolutionary implications, DNA exchange between co-colonizing strains can have important clinical and epidemiological consequences, not only because it has been shown in animal models of disease that minor genetic differences can induce

significant changes in the virulence of a strain (Hiller *et al.*, 2011), but also because this genetic exchange has played a role in the development and evolution of antibiotic resistance (Dowson *et al.*, 1989). The occurrence of capsule switching events is also of importance as it can have an impact on the virulence of strains and, in the context vaccine effectiveness, can result in the emergence of vaccine escape recombinants (Brueggemann *et al.*, 2007). In the same context, understanding co-colonization is critical to monitor the extent of serotype replacement by minor serotypes when vaccination targets the most abundant ones (Lipsitch, 1999; Lipsitch, 2001; Spratt & Greenwood, 2000).

Another important clinical implication is the increasing evidence that co-colonization might be associated with higher colonization densities. Brugger et al (Brugger *et al.*, 2010) showed that colonization density was significantly higher in nasopharyngeal swabs containing multiple pneumococcal strains, compared to swabs with a single strain. Accordingly, Margolis et al (Margolis *et al.*, 2010) have shown, in a neonatal rat model, that sequential colonization with two pneumococcal strains results in increased colonization densities to allow the co-existence of the pulsed and the established strains. This is of great importance due to the increasing interest in using nasopharyngeal colonization density as a diagnostic tool for pneumonia (Albrich *et al.*, 2012). Moreover, a study that assessed co-colonization events in samples from healthy children and children with acute respiratory infection has shown that the latter group was significantly more co-colonized (Dhoubhadel *et al.*, 2014).

Epidemiology

The epidemiology of co-colonization is generally unknown. There are several surveillance studies that report colonization by more than one strain in the same host, but the majority of these studies are not comparable since they have used different

methods of detection and most have not systematically looked at co-colonization (for a review see (O'Brien & Nohynek, 2003)).

Studies that used the same detection method (serotyping of multiple colonies) suggest that co-colonization prevalence is highly variable across geographical settings, ranging between 3% and 36% (Gratten *et al.*, 1989; Hansman & Morris, 1988; Ussery *et al.*, 1996). Also, there is some evidence that co-colonization prevalence in a given setting might be associated with the carriage levels in that setting, *i.e*., settings with higher carriage prevalence will have higher co-colonization levels (Brugger *et al.*, 2010; Kandasamy *et al.*, 2015; Turner *et al.*, 2011).

There is little data on the effect of vaccination on co-colonization. Brugger *et al.* (Brugger *et al.*, 2010) have used a highly sensitive method to detect co-colonization in nasopharyngeal samples collected between 2004 and 2009, spanning PCV7 introduction in Switzerland. They concluded that the rate of co-colonization was similar before and after the introduction of PCV7 and that it was associated with younger age, as children younger than 2 years of age were significantly more co-colonized. This study has also shown that co-colonization rates are independent of the vaccination status of the child, which is in disagreement with the results obtained in Portugal, where it was shown that, in the vaccine era, children with four PCV7 doses were significantly less co-colonized than non-vaccinated children (see chapter II).

The quest for the perfect detection method

The first reports on co-colonization were obtained by successive intraperitoneal saliva injections in mice to detect multiple serotypes (Gundel *et al*. (Gundel M, 1933), reviewed in (Shak *et al.*, 2013)). Using this strategy, Gundel *et al*. reported a 73% rate of co-colonization. Although impractical, this methodology was the basis for the standard approach of using enrichment steps of the nasopharyngeal sample, followed

by serotyping of several colonies, to detect co-colonization. The latter strategy was used for several years and constitutes the basis of most reports on co-colonization (Barker *et al.*, 1989; Gratten *et al.*, 1989; Hansman & Morris, 1988; Huebner *et al.*, 2000; Ussery *et al.*, 1996). Despite being the most straightforward approach for detection of co-colonization, serotyping of multiple colonies has proved to be of little value due to its low sensitivity to detect less abundant serotypes in a nasopharyngeal sample. Huebner at al. (Huebner *et al.*, 2000) have shown that, to have a 95% chance of detecting a serotype present in a sample in a relative abundance of 1%, one would need to serotype 299 colonies. This is highly demanding in feasibility and cost.

For the reasons mentioned above, several alternative serotyping methods have been developed, taking into consideration the ability to detect co-colonization. The criteria to define a good detection method rely on the ability of that method to (i) detect minor serotypes with high specificity, (ii) detect serotypes directly from the nasopharyngeal specimen, (iii) detect all known serotypes, (iv) distinguish pneumococci from closely related species, (v) be quantitative, and (vi) be affordable (Satzke *et al.*, 2012; Satzke *et al.*, 2014).

These methods can be divided into phenotypic and genotypic and some are variants of the same technology (Table I).

Most phenotypic methods are either bead-based (Park *et al.*, 2000; Sheppard *et al.*, 2011; Whaley *et al.*, 2010) or blot-based immunoassays (Bogaert *et al.*, 2004c; Bronsdon *et al.*, 2004). The blot-based methods have the advantage of being cost-effective because they use highly diluted typing sera but they are technically demanding and can be of difficult interpretation due to cross-reactions. The bead-based methods are technically demanding and of difficult implementation, as they require very expensive equipment and extensive optimization.

Turner *et al.* (Turner *et al.*, 2011) have also developed a phenotypic method based on latex agglutination. This method uses an enrichment step of the nasopharyngeal specimen according to the WHO recommendations and resuspension of the primary selective growth on a saline solution to be tested against all available typing sera, allowing the detection of virtually all capsular types or groups. The authors were able to detect minor serotypes that corresponded to down to 25% of the total pneumococcal population.

The genotypic methods include PCR-based methods, including real-time PCR, and microarray-based methods. Their main advantage is their higher sensitivity, when compared to phenotypic methods, which enables the detection minor serotypes present in a very low abundance that could be below the culturable limit.

Several multiplex-PCR systems were developed that are able to detect co-colonization, although all of them require a previous enrichment of the nasopharyngeal specimen (Morais *et al.*, 2007; Rivera-Olivero *et al.*, 2009; Yu *et al.*, 2011). These methods have the limitation of having low discriminative power in closely-related capsular types, of not being quantitative and the fact that most of them target a limited number of capsular types. They present high sensitivity and are of straightforward implementation and execution.

Real-time PCR based methods have also been developed (Azzari *et al.*, 2010; Pimenta *et al.*, 2012). These methods are extremely sensitive but are limited by the low discriminative power in closely-related capsular types and by the optimization difficulties for multiplexing. Azzari *et al.* (Azzari *et al.*, 2010) have shown, however, the main advantage of this approach, by demonstrating its ability to detect co-colonization directly from the nasopharyngeal sample with a four times higher sensitivity than sequential multiplex PCR. Another key advantage of this method is the fact that it is quantitative.

Table I. Summary of advantages and disadvantages of methods for detection of co-colonization.

Method	Main advantages	Main disadvantages	References
Phenotypic methods			
Blot-based	Low cost because use diluted typing sera	Require culture step Technically demanding Interpretation is subjective Lack of specificity due to cross reactions	(Bogaert *et al.*, 2004c; Bronsdon *et al.*, 2004; Fenoll *et al.*, 1997)
Bead-based	Can target capsular polysaccharide or PCR product Good sensitivity	Require culture step Technically demanding Require expensive equipment	(Lal *et al.*, 2005; Park *et al.*, 2000; Sheppard *et al.*, 2011; Whaley *et al.*, 2010)
Latex agglutination	High sensitivity and specificity Easy interpretation	Requires culture step Commercial latex kits are expensive	(Slotved *et al.*, 2004; Turner *et al.*, 2011)
Genotypic methods			
Multiplex conventional PCR	Detection of non-viable organisms Can be applied directly to NP specimen (with lower sensitivity than real-time PCR) High throughput	Low discriminative power in closely-related capsular types Not quantitative Presence of capsular genes in *Streptococcus spp*. can induce false positive results	(Morais *et al.*, 2007; Rivera-Olivero *et al.*, 2009; Yu *et al.*, 2011)
Real-time PCR	Extremely sensitive Quantitative Detection of non-viable organisms Can be applied directly to NP specimen High throughput	Low discriminative power in closely-related capsular types Presence of capsular genes in *Streptococcus spp*. can induce false positive results	(Azzari *et al.*, 2010; Pimenta *et al.*, 2012)

Table I. *Continued*.

Method	Main advantages	Main disadvantages	References
Genotypic methods (*cont.*)			
plyNCR-RFLP	High sensitivity and specificity Detection of non-viable organisms High throughput	Requires culture step Requires additional step for serotype assignment Requires high level of technical expertise	(Brugger *et al.*, 2009; Brugger *et al.*, 2010)
Microarray	Highly sensitive Detection of non-viable organisms Capacity to detect all capsular types and other markers (virulence factors, antibiotic resistence genes, etc) Measures relative abundance of serotypes	Require culture step Expensive equipment required Require high level of technical expertise Difficulty in distinguishing between closely-related species, although with capacity to increasing number of targets per species	(Hinds *et al.*, 2010; Tomita *et al.*, 2011; Turner *et al.*, 2011; Wang *et al.*, 2007)

NP – nasopharyngeal. Adapted from (Satzke *et al.*, 2014) with information collected in the cited references and in (Satzke *et al.*, 2012).

The main drawback of both conventional and real-time PCR based methods is the possible occurrence of false positive results associated with the fact that these methods use as target fragments of capsular genes that can potentially be present in other inhabitants of the nasopharynx, such as closely related *Streptococcus spp*. (Carvalho Mda *et al.*, 2013).

Brugger *et al.* (Brugger *et al.*, 2009) have developed a PCR-based method which takes advantage of a highly conserved and specific genetic region within the pneumococcal species, adjacent to the pneumolysin gene (*ply*), which encodes for a major virulence factor and is highly specific for pneumococcus. This variable noncoding region adjacent to the *ply* gene (*ply*NCR) is amplified by PCR and the PCR product is then digested for restriction fragment length polymorphisms (RFLP). The restriction is performed with four enzymes used independently and the samples are analyzed by capillary electrophoresis for exact size assessment of the digestion fragments. When the sum of the restriction fragments exceeds the size of the undigested product (c.a. 1400 bp), there is evidence that more than one pneumococcal strain exist in the sample. This method has high throughput but requires expensive equipment and reagents and a fairly high level of technical expertise, as interpretation of results can be misguided by incomplete digestion or presence of closely-related *Streptococcus spp*. The greatest disadvantage of this method is related to the fact that it does not discriminate the serotypes of the co-colonizing strains. For this reason, it has to be coupled with another genotypic method that has this ability.

Genotypic methods based on comparative genomic hybridization (CGH) have also been developed (Hinds *et al.*, 2010; Tomita *et al.*, 2011; Turner *et al.*, 2011; Wang *et al.*, 2007). However, to our best knowledge, only the microarray developed by Hinds *et al.* (Hinds *et al.*, 2010; Turner *et al.*, 2011) has the ability to detect all capsular types described up to now. In this method each capsular gene is targeted by ten 60-mer oligonucleotide reporters resulting in a very robust method for the assignment of

capsular types. As detection is based on fluorescently labeled oligomers, this microarray is able to quantify the relative abundance of each capsular type in the total of pneumococcal DNA present in the samples. Despite being very sensitive, this method is not suitable for detection directly from the nasopharyngeal specimen, although optimization is ongoing (Satzke *et al.*, 2012). The main drawback of this method is, however, the requirement of very expensive reagents and equipment and high level of technical expertise.

Due to the existence of all these different approaches and to the increasing relevance attributed to co-colonization in vaccine surveillance studies, an initiative was undertaken under the scope of the PneuCarriage project in which 20 methods were compared for their ability to detect co-colonization with a good cost/benefit ratio. This assessment is being done by using a reference collection of spiked samples mixed with real samples, in which methods with the best performance in detecting minor serotypes with good positive predictive value will be selected for a second round that consists in the analysis of 200 field samples (Satzke *et al.*, 2012). First reports announced that four methods were selected for the second stage of evaluation: the microarray developed by Hinds *et al*. (Hinds *et al.*, 2010; Turner *et al.*, 2011), the latex agglutination method developed by Turner *et al.* (Turner *et al.*, 2011), the plyNCR-RFLP coupled with multiplex PCR, developed by Brugger *et al.* (Brugger *et al.*, 2009), and the multiplex PCR and real-time PCR developed by Azzari *et al*. (Azzari *et al.*, 2010).

Co-colonization determinants

Co-colonization has been known to occur for many years, although the factors determining its occurrence and the interactions that occur between strains inside the host only recently have begun to be addressed.

Several theoretical models have been used to predict which are the determinants of co-colonization and strain interactions, all of them considering the capsular type as the decisive factor to distinguish between strains (Auranen *et al.*, 2009; Cobey & Lipsitch, 2012; Zhang *et al.*, 2004). These studies have resulted in the formulation of important premises about between-strain competition and carriage of multiple serotypes: co-colonization does not seem to accelerate clearance (Auranen *et al.*, 2009), established colonization with one serotype reduces risk of acquisition of a second serotype (Auranen *et al.*, 2009), serotype-specific immunity stabilizes competition, and acquired immunity to non-capsular antigens reduces fitness differences between strains, being relevant for competition (Cobey & Lipsitch, 2012; Zhang *et al.*, 2004). Notwithstanding, these theoretical principles have not been sufficiently addressed experimentally.

Experimental and epidemiological data on this subject is scarce and the bacterial factors that might affect this phenomenon are generally unknown. In the following sections information regarding some bacterial factors that might have an impact on co-colonization and that are relevant in the context of this thesis will be presented.

The contributions of the capsule and genetic background to (co)colonization

The ability of a strain to colonize an individual is intrinsically related to its ability to resist clearance. With the objective of determining the invasive potential of different serotypes, Sleeman *et al.* (Sleeman *et al.*, 2006) have shown that less invasive serotypes are carried for longer periods. Most studies that have addressed which factors contribute to the a success of a strain in colonization have focused on the role of the capsule, as it is the main target of the immune system in the clearance process and was the first factor shown to be important for *S. pneumoniae* virulence. The

capsule prevents clearance and aggregation, affects colonization and adherence, helps the pneumococcus to survive in the lungs and spread to the bloodstream, and contributes to antibiotic tolerance. Pneumococci regulate the amount of capsular material produced during colonization and invasion. Transparent capsules (thin) are favored during initial colonization stages to promote adherence to host epithelial cells, while opaque capsules (thick) are favored during invasion to resist complement-mediated opsonophagocytosis (Fernebro *et al.*, 2004; Lysenko *et al.*, 2005; Mac & Kraus, 1950; Morona *et al.*, 2004; Nelson *et al.*, 2007; Weiser *et al.*, 1994).

Weinberger *et al*. (Weinberger *et al.*, 2009) have shown that the structure of the capsular polysaccharide can predict serotype prevalence. These authors and others have shown that serotypes with capsule structures with more carbon in their polysaccharide repeat unit required more energy for capsule production, displaying thinner capsules. Therefore, these serotypes were less resistant to phagocytosis and clearance, which correlated with their lower prevalence (Hathaway *et al.*, 2012; Weinberger *et al.*, 2009). Other capsule-related properties have been studied to explain the influence of the capsular type on pneumococcal colonization, such as the surface charge. Li *et al.* (Li *et al.*, 2013) have shown that the surface charge correlates with the prevalence of serotypes in colonization, as more negatively charged capsular types are more prevalent than less charged capsular type and capsules more negatively charged were shown to repel host immune cells that contribute to clearance, such as neutrophils and macrophages.

Several epidemiological studies have shown that there is high variability in strains of the same serotype (Brueggemann *et al.*, 2003; Hanage *et al.*, 2005; Sá-Leão *et al.*, 2011; Sandgren *et al.*, 2004) and this observation is supported by the increasing available genomic data (Chewapreecha *et al.*, 2014; Hakenbeck *et al.*, 2001; Hiller *et al.*, 2007). This genetic variation is associated with the capsular type to some extent

(http://www.mlst.net/). For this reason, the differences observed between capsular types might be partially related to the genetic background of the strains.

Moreover, several capsule-independent factors have been shown to interfere with complement-mediated immunity (reviewed in (Jarva *et al.*, 2003)), highlighting the importance of the genetic background of the bacteria to avoid clearance and, in this way, promote colonization. Yuste *et al.* (Yuste *et al.*, 2009) have shown that loss of the pneumococcal surface protein C (PspC) affected differently pneumococcal strains regarding complement activation by C3 deposition, and this effect was dependent on the strain background. Pneumococcal histidine triad proteins have also been shown to promote complement evasion on a background-dependent manner (Melin *et al.*, 2010). Hyams *et al*. (Hyams *et al.*, 2011) also demonstrated that genetically distinct *S. pneumoniae* strains with the same capsular serotype vary in their sensitivity to complement mediated immunity, re-enforcing the importance of the genetic background to colonization.

Another important aspect that promotes colonization is the ability of strains to produce and survive in a biofilm, as there is growing evidence that this lifestyle is important for colonization (Hoa *et al.*, 2009; Kania *et al.*, 2008; Marks *et al.*, 2012a; Psaltis *et al.*, 2007; Sanclement *et al.*, 2005). This ability is not solely dependent on the serotype of the strains (Blanchette-Cain *et al.*, 2013) and several non-capsular bacterial factors have been shown to be important for biofilm formation (Blanchette-Cain *et al.*, 2013; Sanchez *et al.*, 2010; Vidal *et al.*, 2013). On the other hand, although the type of capsule does not seem to be important for biofilm formation, the modulation of the capsular expression was shown to be critical, with less encapsulated variants being important for the initial stages of attachment (Domenech *et al.*, 2009).

Overall, both the capsule and the genetic background are important factors to promote colonization and, hence, they must impact on carriage of multiple strains by the same

host, even if indirectly. The contribution of these bacterial properties has never been addressed experimentally in the context of co-colonization. The experiments performed by Lipsitch *et al.* (Lipsitch *et al.*, 2000), previously mentioned, have shown that competition *in vivo* occurs in a strain dependent manner but the contribution of the capsule or the genetic diversity of the strains has not been sufficiently addressed. Very recently, Trzcinski *et al*. (Trzcinski *et al.*, 2015) have shown in co-infection experiments in a mouse model, using clinical strains and isogenic variants of the same capsular types, that there was a stable hierarchy of capsular types colonizing the animals in different conditions. Moreover, the authors were able to correlate this hierarchy with serotype specific fitness predicted by capsule-dependent properties, such as capsule synthesis metabolic cost and surface charge (Li *et al.*, 2013; Weinberger *et al.*, 2009). These results suggest a significant role of the capsule on strains competitive abilities and fitness when interacting with strains of other serotypes.

Chemical war I - competence-mediated fratricide

The pneumococcus is naturally transformable, which means it can acquire external DNA and incorporate it into its own chromosome. This ability of the pneumococcus was first shown by Griffith in 1928 (Griffith, 1928) but the first insights on pneumococcal competence were provided only in the 1960s by Tomasz and Hotchkiss, first by describing that competence induction is dependent on cell density (Tomasz & Hotchkiss, 1964) and then by showing that it is controlled by a hormone-like cell product, later identified as the competence stimulating peptide (CSP) (Tomasz, 1965).

Induction of competence is tightly regulated by the *comABCDE* genes, that encode for the signaling peptide CSP (*comC*), a dedicated ABC transporter (*comAB*) and a two component regulatory system (*comDE*) composed by a cognate histidine kinase

receptor (*comD*) and a response regulator (*comE*) (Havarstein *et al.*, 1995a; Havarstein *et al.*, 1996; Havarstein, 1998).

CSP is synthesized inside the cell as a 24-aa pre-peptide that is cleaved upon secretion by the dedicated proteolytic ABC-transporter (Havarstein *et al.*, 1995b), being secreted as a 17-aa peptide (Havarstein *et al.*, 1995a). Accumulation of CSP in the medium stimulates the ComDE two component sytem (Pestova *et al.*, 1996). The ComD receptor autophosphorylates upon binding to CSP and transphosphorylates the response regulator ComE (Havarstein *et al.*, 1996). ComE activates transcription of the *comABCDE* genes, *comX*, which encodes the sigma factor that controls activation of late competence genes (Lee & Morrison, 1999), and *comM*, that encodes a membrane immunity protein expressed on the surface of competent cells only (Havarstein *et al.*, 2006).

There are up to 124 genes that are regulated by competence (Dagkessamanskaia *et al.*, 2004; Peterson *et al.*, 2004), among which are the *cibABC*, encoding for a two peptide bacteriocin (*cibAB*) and an immunity protein (*cibC*), *lytA,* the major autolysin, and *cbpD,* an autolytic amidase (Guiral *et al.*, 2005). The CibAB has been shown to be indispensible for the lysis of non-competent cells through a mechanism that requires contact between competent and non-competent cells and also the presence of hydrolases (LytA, LytC and CbpD), as CibAB by itself is not enough to induce bacterial lysis of non-competent cells. This CibAB-mediated lysis of non-competent cells promoted by competent ones was shown to be important to explain lysis in solid media and was designated by allolysis (Guiral *et al.*, 2005). Added to the protection from CibAB by CibC, competent cells are also protected from their over-expressed hydrolases CbpD, LytA and LytC by the immunity protein ComM, which was shown to protect competent cells from their own lysins in liquid culture (Havarstein *et al.*, 2006). Contrary to CibC that protects competent cells exclusively from the action of CibAB, the specificity of ComM is not known yet. It has not been established yet whether this protein

protects cells specifically against the action of CbpD, or if the immunity is directed against LytA and/or LytC (Figure 2).

These competence-mediated lysis mechanisms target non-competent cells, regardless of whether they are isogenic - as would be the case in a monoculture - or non-isogenic to the competent ones - in the case of a co-culture of different strains - and is generally designated by fratricide. In either case, lysis requires the co-existence of competent and non-competent cells.

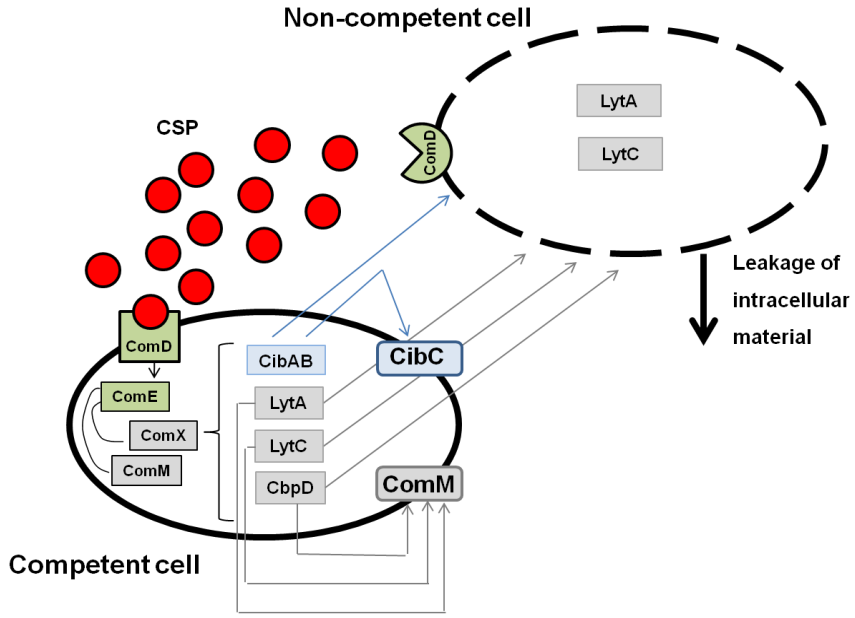

Figure 2. Mechanisms of competence-mediated fratricide. In a population of mixed pherotypes, the competence-race is won by the pherotype that accumulates higher concentration of its own CSP in the medium. Sensing of CSP induces competence in the population that expresses that cognate ComD receptor, resulting in the cascade of events described in the text. Adapted from (Guiral *et al.*, 2005; Johnsborg *et al.*, 2008; Johnsborg & Havarstein, 2009).

The pheromone CSP is prone to allelic variation (Havarstein *et al.*, 1995a; Whatmore *et al.*, 1999) and this can contribute to the occurrence of fratricide when strains of different pherotypes come across each other, as the ComD receptor only recognizes its cognate CSP type. Two dominant allelic variants have been identified in the pneumococcal

population – CSP1 and CSP2 (Pozzi *et al.*, 1996; Ramirez *et al.*, 1997; Whatmore *et al.*, 1999).

The impact of pherotype on within-host competition has not been fully addressed. If on one hand it has been shown that the type of CSP restricts competence within pherotypes, which is important for fratricide (Guiral *et al.*, 2005; Johnsborg *et al.*, 2008), on the other hand, to what extent these mechanisms are important for the co-existence of different pneumococci in the same host has not been clarified yet. A number of studies that used biofilm models to simulate dual-strain carriage have shown that fratricide occurs in those conditions and that it is important for gene transfer between strains (Carrolo *et al.*, 2014; Marks *et al.*, 2012b; Wei & Havarstein, 2012). Marks *et al.* (Marks *et al.*, 2012b) have shown that competence genes are up-regulated in conditions that mimic the nasopharyngeal environment (lower temperature, limited nutrient availability and interaction with epithelial cells) and also that competence is induced during dual-strain carriage *in vivo*. None of these studies has, however, addressed the question of whether the co-existence of strains of different pherotypes could be impaired by this induction of competence.

Using an epidemiological approach, Vestrheim *et al*. (Vestrheim *et al.*, 2011) have looked at pairs of co-colonizing strains to evaluate the impact of pherotype on the co-existence of pneumococci in the nasopharynx. The authors showed that pneumococcal pherotypes often co-exist in the nasopharynx and suggested that the impact of competence induced fratricide on competition is limited. However, this study used co-colonized samples identified on the basis of colony morphology, a method shown to underestimate co-colonization events and to introduce a bias in the serotype distribution of co-colonizing strains (for a reference see Chapter IV).

In line with the observation of Vestrheim *et al.*, Carrolo et al. (Carrolo *et al.*, 2014) have shown that, within biofilms, intra-pherotype fratricide prevails over inter-pherotype

fratricide, probably due to the spatial organization of strains of different pherotypes within the biofilm. This hypothesis is supported by the observation that physical contact between competent and non-competent cells might be needed for fratricide to occur (Havarstein *et al.*, 2006).

Despite this, at what extent competence-mediated fratricide impacts on pneumococcal co-existence in the nasopharynx remains to be understood.

Chemical war II - bacteriocin secretion

Microbes in general are equipped with a large assortment of defense-attack systems: broad-spectrum classical antibiotics, metabolic by-products, lytic agents, several types of protein exotoxins and bacteriocins, which are loosely defined as biologically active proteins/peptides with a bactericidal activity (reviewed in (Riley & Wertz, 2002; Tagg *et al.*, 1976)). Among all existing microbial defense-attack systems, bacteriocins seem to be the preferential weapon as these toxins have been found in all Phyla of Bacteria and have been described to be produced by some Archaea members (Klaenhammer, 1988; Torreblanca *et al.*, 1994). The key difference between bacteriocins and traditional antibiotics is that, contrary to antibiotics, bacteriocins have a relatively narrow killing spectrum and are only toxic to bacteria closely related to the producing strain (Eijsink *et al.*, 2002). It is, in fact, this property that is on the basis of the increasing interest of bacteriocins as “designer drugs” to combat bacterial infections, in an attempt to decrease the pressure for the selection of antibiotic resistance and the collateral killing of commensal species (reviewed in (Cotter *et al.*, 2012)).

In general, bacteriocins are short hydrophobic and/or amphipatic peptides, ribosomally synthesized as an inactive preptide with an N-terminal leader sequence and a C-terminal propetide. The leader sequence targets bacteriocins to a dedicated transporter

and keeps bacteriocins in an inactive form until they are secreted (Nissen-Meyer *et al.*, 2009). There are several types of bacteriocins that are divided in four classes: i) lantibiotics (modified bacteriocins), ii) unmodified peptides, iii) large proteins, and iv) cyclic peptides (Heng & Tagg, 2006). The bacteriocins discussed in the context of this thesis belong to the second class.

All bacteriocin producing strains are immune to their own product through a mechanism not well understood yet. For most bacteriocins the immunity gene(s) is located either in the same operon as the bacteriocin gene or in close vicinity.

Genomic analysis of eleven sequenced *S. pneumoniae* strains has identified nine putative bacteriocin-like gene clusters, one cluster of class I and the remaining eight of class II. These clusters were shown to vary in presence and content among those strains (Majchrzykiewicz, 2011). Of these, only two class II gene clusters have been shown to induce antimicrobial activity: the *blp (bacteriocin-like peptide)* and *cib (competence-induced bacteriocins)* loci. The role of the *cib* locus was described previously in this chapter. In addition, very recently Bogaardt *et al.* (Bogaardt *et al.*, 2015) have described a cassette encoding a circular bacteriocin that was present in one third of a diverse collection of over 300 sequenced pneumococci. These authors have shown that this cassette had a genetic organization similar to other circular bacteriocin cassettes well characterized in other species. However, no experimental data on the antimicrobial activity of this cassette was presented yet.

The *blp* - bacteriocins

In *S. pneumoniae*, the *blp* locus encodes a number of bacteriocins that have been implicated in intraspecific competition in a murine model of colonization (Dawid *et al.*, 2007). These bacteriocins are characterized by the presence of a conserved N-terminal leader peptide, followed by a typical double-glycine motif where the prepeptide is

cleaved upon secretion (Bogaardt *et al.*, 2015; Dawid *et al.*, 2007; de Saizieu *et al.*, 2000; Son *et al.*, 2011).

The *blp locus* (Figure 3) encodes a signalling peptide, BlpC, a two component system, BlpRH, constituted by an histidine kinase cognate receptor (BlpH) and a response regulator (BlpR), an ABC transporter, BlpAB, and the bacteriocin and immunity region (BIR), which contains bacteriocin peptides and their cognate immunity proteins, typically co-transcribed (Dawid *et al.*, 2007; Reichmann & Hakenbeck, 2000; Son *et al.*, 2011). In addition, downstream to the BIR, the locus contains also conserved proteins thought to be involved in immunity (*blpYZ* and *SPO547*) (Dawid *et al.*, 2007; de Saizieu *et al.*, 2000; Lux *et al.*, 2007; Son *et al.*, 2011).

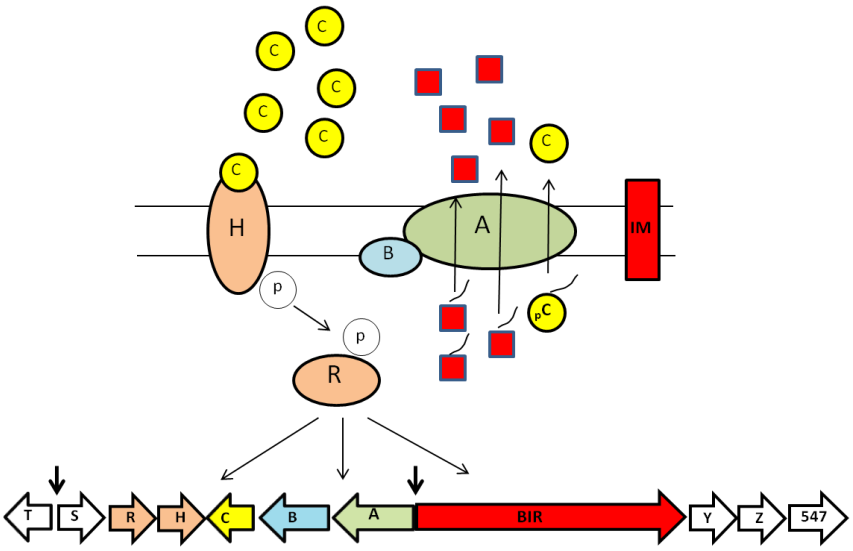

Figure 3. *blp* locus and model of locus activation. BlpC is secreted by the ABC transporter BlpAB. At high cell-density, BlpC accumulates extracellularly and is sensed by its cognate receptor BlpH, which in turn activates the response regulator BlpR. BlpR up-regulates the transcription of the entire locus at the sites marked by the arrows above the locus, including the BIR. Bacteriocin peptides are also secreted by BlpAB and immunity proteins are expressed at cell surface. Adapted from (Son *et al.*, 2011).

Bacteriocin secretion is tightly regulated by the two component system. Activation of the system occurs upon binding of the signalling peptide BlpC to its cognate BlpH receptor, after secretion of BlpC by the dedicated ABC transporter BlpAB (Dawid *et al.*,

2007; Reichmann & Hakenbeck, 2000). BlpC is secreted at basal levels but once a critical threshold is reached, BlpR is phosphorilated, which has been shown to result in the upregulation of the entire locus, including *blpC* and the BIR. Bacteriocin peptides are also secreted by the ABC transporter BlpAB and immunity proteins are expressed at cell membrane (de Saizieu *et al.*, 2000).

Little is known of other regulation mechanisms of *blp* bacteriocin secretion other than the cognate two component system BlpRH. It was shown that the ComDE regulatory system is able to upregulate, likely indirectly, some of the *blp* genes (*blpZYA*, involved in the production of transport and immunity proteins) (Peterson *et al.*, 2004). Also, the conserved protease HtrA has been shown to control activation of the locus and bacteriocin secretion by limiting BlpC processing and secretion. In this way, locus activation is restricted to high cell density, preventing unnecessary and energetically costly bacteriocin secretion at low cell density (Dawid *et al.*, 2009; Kochan & Dawid, 2013).

The *blp* locus is characterized by high diversity. The *blpA* gene, encoding the ABC transporter, contributes for this variability, as it can correspond to a single open reading frame (ORF) or it can be divided in smaller ORFs due to the presence of frameshift mutations or deletions. Son *et al*. (Son *et al.*, 2011) have described an interruption of the ORF by a widely conserved 4 bp repeat insertion that renders strains with an otherwise intact *blp* locus unable to secrete both the peptide pheromone and the bacteriocin peptides. Despite their defective transport system, these strains are able to recognize exogenous peptides, resulting in the expression of immunity proteins without the cost of peptide secretion, this being the reason that led the authors to designate these strains as “cheaters”. Cheater strains are at risk upon encounter with a strain that secretes a BlpC peptide different from the one they can sense. In the same study, the authors refer to the presence of other loss of function mutations and deletions in the *blpA* gene, although no additional details are provided. Also in the context of *blpA*

disruption, Kjos *et al.* (Kjos *et al.*, 2015) have shown, very recently, a correlation between the *com* and *blp* systems, demonstrating that a strain with a disrupted *blpA* (D39) was able to activate its *blp* locus using *comAB* as a transport system. Whether or not this is a general behaviour in pneumococci remains to be assessed, but, if this is the case, than there would be no cheater strains in the population, with all the ecological implications associated with this observation.

Another source of variability of the *blp* locus is the peptide pheromone, *blpC*, for which at least 5 allelic variants have been described: $blpC_{T4}$, $blpC_{R6}$, $blpC_{6A}$, $blpC_{P164}$, and $blpC_{P155}$, the first four being dominant in the population (de Saizieu *et al.*, 2000; Pinchas *et al.*, 2015; Reichmann & Hakenbeck, 2000; Son *et al.*, 2011). Despite the correspondence typically found between signalling peptide and receptor in this type of regulatory systems, the allelic variation in the pheromone receptor, *blpH,* is higher, due to the existence of naturally occurring chimeras for this gene. Pinchas *et al.* (Pinchas *et al.*, 2015) have found that $blpH_{6A}$ has three sub-alleles, whose gene products share 98% sequence identity: $blpH_{6A.1}$, $blpH_{6A.2}$ and $blpH_{6A.3}$. These authors have shown that the BlpH variants have different specificities to the BlpC pheromone: $BlpH_{P164}$ and $BlpH_{R6}$ respond only to their cognate BlpC pheromone, while $BlpH_{6A.1}$ and $BlpH_{6A.2}$, respond to both $BlpC_{T4}$ and $BlpC_{6A,}$ although the sensitivity of the receptor to the non-cognate pheromone is lower. $BlpH_{6A.3}$ is a $BlpH_{P164/6A}$ chimera and responds to both $BlpC_{P164}$ and $BlpC_{6A}$, although with higher sensitivity to the non-cognate pheromone $BlpC_{P164.}$

The bacteriocin immunity region (BIR), enclosing genes that code for bacteriocins and their cognate immunity proteins, typically co-transcribed, is also highly variable, both in size and content. At least sixteen putative bacteriocin peptides have been described so far, some of which present allelic variability (Bogaardt *et al.*, 2015; Son *et al.*, 2011). This variability may in inhibition upon the encounter of two strains, even if they can

sense the same signalling peptide, as cells will only express immunity proteins against the bacteriocin peptides that they secrete.

The mode of action of these bacteriocins is not elucidated yet in *S. pneumoniae*, but the most plausible mode of action is through pore formation on the citoplasmic membrane of susceptible cells, as described for most class II bacteriocins (Hechard & Sahl, 2002; Majchrzykiewicz, 2011).

The putative immunity proteins present in this region are membrane proteins but their function and mode of action has not been experimentally demonstrated yet. Some cassettes contain also a CAAX amino-terminal protease (Bogaardt *et al.*, 2015; Dawid *et al.*, 2007; de Saizieu *et al.*, 2000). CAAX proteases have been proposed to be involved in immunity against bacteriocins but they remain poorly studied in prokaryotes (Kjos *et al.*, 2010; Pei & Grishin, 2001). For this reason little is known about the mechanism of protection of these proteins. They are putative membrane-bound metalloproteases thought to recognize and protect, most likely by proteolytic cleavage, a common receptor to the bacteriocin they protect from (Lux *et al.*, 2007; Majchrzykiewicz, 2011; Nes & Hole, 2000). The *blp* locus contains two additional CAAX proteases at the end of the cassette, downstream of the BIR, BlpY and BlpZ. BlpY has been shown to be essential for immunity and bacteriocin activity (Lux *et al.*, 2007).

The importance of *blp* bacteriocins has been demonstrated in a murine model of colonization. Dawid *et al*.(Dawid *et al.*, 2007) have used wild type and bacteriocin-deleted mutants in dual colonization experiments and have observed that the production of bacteriocins by the wild type strains was able to inhibit the growth of the deleted mutants. These results showed that the locus is active *in vivo* and is able to play a role in the polymicrobial environment of the nasopharynx. How extensive this role is remains to be addressed.

Aim of the thesis

Although pneumococcal co-colonization has been known for several decades, it remains poorly studied. Its detection is important for monitoring vaccine effectiveness since it allows the distinction between unmasking and serotyping replacement phenomena. Also, colonization with multiple serotypes is thought to be the driving force for competition and evolution in the species, either by competition for nutrients or by promoting gene exchange between strains.

The work developed under the scope of this thesis aimed at (i) determining the prevalence of co-colonization among Portuguese children and evaluating the impact of the introduction of pneumococcal conjugate vaccines on its dynamics; (ii) identifying significant positive or negative associations between pneumococcal strains; and (iii) exploring the molecular mechanisms that could underlie significant associations, focusing on bacterial-related properties such as the capsule, the genetic background, competence mediated-fratricide, and bacteriocin secretion.

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Chapter II

Decrease in pneumococcal co-colonization following vaccination with the seven-valent pneumococcal conjugate vaccine

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Contributions:

C. Valente was responsible for all experimental work with the exception of the majority of the comparative genomic hybridization experiments, which were performed by K. Gould and analyzed by J. Hinds. F. Pinto performed the statistical analyses.

Summary

Understanding the epidemiology of pneumococcal co-colonization is important for monitoring vaccine effectiveness and the occurrence of horizontal gene transfer between pneumococcal strains. In this study we aimed to evaluate the impact of the seven-valent pneumococcal conjugate vaccine (PCV7) on pneumococcal co-colonization among Portuguese children. Nasopharyngeal samples from children up to 6 years old yielding a pneumococcal culture were clustered into three groups: pre-vaccine era (n=173), unvaccinated children of the vaccine era (n=169), and fully vaccinated children (4 doses; n=150). Co-colonization, serotype identification, and relative serotype abundance were detected by analysis of DNA of the total bacterial growth of the primary culture plate using the *ply*NCR-RFLP method and a molecular serotyping microarray-based strategy. The *ply*NCR-RFLP method detected an overall co-colonization rate of 20.1%. Microarray analysis confirmed the *ply*NCR-RFLP results. Vaccination status was the only factor found to be significantly associated with co-colonization: co-colonization rates were significantly lower (p=0.004; Fisher's exact test) among fully vaccinated children (8.0%) than among children from the pre-PCV7 era (17.3%) or unvaccinated children of the PCV7 era (18.3%). In the PCV7 era there were significantly less non-vaccine type (NVT) co-colonization events than would be expected based on the NVT distribution observed in the pre-PCV7 era (p=0.024). In conclusion, vaccination with PCV7 resulted in a lower co-colonization rate due to an asymmetric distribution between NVTs found in single and co-colonized samples. We propose that some NVTs prevalent in the PCV7 era are more competitive than others, hampering their co-existence in the same niche. This result may have important implications since a decrease in co-colonization events is expected to translate in decreased opportunities for horizontal gene transfer, hindering pneumococcal evolution events such as acquisition of antibiotic resistance determinants or capsular switch. This might represent a novel potential benefit of conjugate vaccines.

Introduction

Streptococcus pneumoniae (the pneumococcus) remains a main cause of morbidity and mortality worldwide (O'Brien *et al.*, 2009). Its ecological niche is the human nasopharynx. Colonization by pneumococcus can occur soon after birth and remains high in the first years of life (Bogaert *et al.*, 2004). Virtually every child is colonized by pneumococcus at some stage in life and each serotype can colonize for several weeks being then replaced by another serotype or reacquired (Gray *et al.*, 1980; Sá-Leão *et al.*, 2008).

Although poorly studied, it has been known for decades that simultaneous carriage of multiple pneumococci (or co-colonization) can occur (Griffith, 1928; Hodges *et al.*, 1946). Co-colonization is an important event for pneumococcal evolution as it represents an opportunity for horizontal gene transfer, the main mechanism of evolution in this species (Barnes *et al.*, 1995; Spratt *et al.*, 2001).

Studies on co-colonization have been hampered by the lack of suitable detection methods. The limited reports available have found co-colonization rates in the range of 5-30% (Brugger *et al.*, 2009; Gray *et al.*, 1980; Hare *et al.*, 2008; Montgomery *et al.*, 1990; O'Brien & Nohynek, 2003; Rivera-Olivero *et al.*, 2009; Sá-Leão *et al.*, 2002). However, most studies have relied on serotyping individual colonies isolated from culture. This approach has low sensitivity, is expensive and time-consuming and is biased to detect only the most abundant serotypes (Hare *et al.*, 2008; Huebner *et al.*, 2000).

In recent years, with the introduction of multivalent pneumococcal conjugate vaccines, there has been a renewed interest in the study of co-colonization since it is important to understand serotype changes among carriers following vaccination, for instance, to distinguish increased acquisition from unmasking phenomena (Lipsitch, 1999).

Simultaneously, novel approaches for detection of co-colonization have been proposed (Bronsdon *et al.*, 2004; Brugger *et al.*, 2009; da Gloria Carvalho *et al.*, 2010; Rivera-Olivero

et al., 2009; Turner *et al.*, 2011). In particular, Brugger *et. al*. have developed, the *ply*NCR-RFLP method, based on the restriction pattern of a highly conserved DNA region within the pneumococcal species (Brugger *et al.*, 2009). Additionally, Hinds *et al.* developed a molecular serotyping microarray based on genomic DNA hybridization that is able to detect and quantify all serotypes described to date (Hinds *et al.*, 2010).

In Portugal, the seven-valent pneumococcal conjugate vaccine (PCV7) became commercially available in June 2001 and, though the vaccine is not included in the National Immunization Program, it has been widely prescribed as the Portuguese Society of Pediatricians issued recommendation for PCV use among all young children. These included a catch up schedule. Rates of PCV coverage increased gradually since 2001. Estimates from Pfizer based on annual sales and considering 3.5 doses per newborn are as follows from 2001 to 2007: 17%, 32%, 56%, 65%, 63%, 75%, and 79%, respectively. In studies conducted by us, by 2006-2007, c.a. 70% of children (aged up to 6 years old) had received at least one PCV7 dose (Simões *et al.*, 2011). Major serotype shifts have occurred since 2001 both in disease and colonization (Aguiar *et al.*, 2008; Sá-Leão *et al.*, 2009). Although colonization rates have remained stable (Aguiar *et al.*, 2008; Sá-Leão *et al.*, 2009), the effect of vaccination on co-colonization has remained unknown.

In this study, using a combination of the *ply*NCR-RFLP and the molecular serotyping microarray methods, we aimed to gain insights on the prevalence of co-colonization and evaluate potential changes that might have occurred following vaccination with PCV7 in Portugal.

Materials and methods

Study design. Nasopharyngeal (NP) swabs collected from healthy children attending day-care centers in Oeiras, Portugal, were retrospectively selected (Mato *et al.*, 2005; Sá-Leão *et*

al., 2009). In each year, samples were collected in the winter months of January to March. Samples were selected according to the following criteria: (i) swabs were obtained from children aged 18-71 months; (ii) children had not received antibiotic within the month preceding sampling; and (iii) swabs yielded a pneumococcal positive culture upon direct plating within four hours of collection as described below.

The samples were clustered in three groups matched for age and gender: group I included samples from the pre-vaccine era (n=173, collected in 2001); group II included samples collected in the vaccine era from unvaccinated children (n=169, collected in 2006-2007); and group III included samples collected in the vaccine era from fully vaccinated children (i.e., 4 doses, n=150, collected in 2006-2007) (Supplementary Table). This study was nested in a previous one aimed to study the impact of PCV7 on colonization (Sá-Leão *et al.*, 2009). Approval for the initial study was obtained from the Ministry of Education and directors of day-care centers. Signed informed consent was obtained from the parents or guardians of participating children. Samples and questionnaires were attributed a number and all information was treated anonymously.

Isolation of pneumococci and sample preservation. NP samples were collected by pediatric nurses using mini-tip calcium alginate sterile swabs and inoculated directly within 4 hours in a primary selective plate of 5% blood trypticase soy agar containing gentamicin (5 mg/liter) to select for *S. pneumoniae*. Plates were incubated overnight at 37°C under anaerobic conditions, with an optochin disk. On the following day, presumptive pneumococcal colonies exhibiting different morphologies were picked (one colony per morphology) and subcultured. The remaining bacterial lawn was collected and frozen at -80ºC in 1ml Mueller-Hinton broth containing 30% glycerol. On the third day, cultures derived from the isolated colonies were also frozen. Swabs from 2001 were discarded after plating; swabs from 2006 and 2007 were stored frozen.

PCR serotyping. Pure cultures were serotyped by PCR, as described previously (Pai *et al.*, 2006), using primers and conditions available at http://www.cdc.gov/ncidod/biotech/strep/pcr.htm. Strains that could not be typed by PCR were serotyped by the Quellung reaction using commercially available pneumococcal antisera (Statens Serum Institute, Copenhagen, Denmark).

DNA isolation. Total DNA was isolated from 200µl of the primary selective culture frozen stock using the High Pure PCR Template Preparation Kit (Roche Applied Science) according to the manufacturer's instructions.

***ply*NCR-RFLP.** Detection of co-colonization was done as previously described (Brugger *et al.*, 2009). Briefly, the noncoding region between the pneumolysin gene and the preceding hypothetical protein gene was amplified by PCR. The product was separately digested with up to four restriction enzymes: AflIII, ApoI, DdeI, and MseI. A sample was assumed to contain more than one strain whenever the sum of the size of the digestion fragments was higher than the size of the undigested PCR product.

Molecular serotyping microarray. The BµG@S SP-CPSv1.4.0 microarray designed for *S. pneumoniae* molecular serotyping was used following standard protocols previously described (Brugger *et al.*, 2010). Microarray data was statistically analyzed using a Bayesian hierarchical model to determine the serotype, or combination of serotypes, present in the sample and assign their relative abundance (Newton *et al.*, 2011). In the interpretation of co-colonization results, a serotype was classified as dominant if its relative abundance in the sample was ≥70%.

Statistical analysis. Pair-wise associations between co-colonization and vaccination status, time period, age, gender and day-care center were measured. Statistical significance was accessed through Fisher exact tests. A Kolmogorov-Smirnov test was applied to compare

age distribution between multiple and single carriers. Associations were considered significant if $p<0.05$.

Multivariate logistic regression models were used to detect associations with co-colonization, using vaccination status, time period, age, gender and day-care center as independent variables. As day-care center was a categorical variable with more than two categories, 14 dummy variables (binary variables for all but one day-care centre) were constructed and used.

Models were adjusted with all independent variables simultaneously and through backward and forward stepwise variable selection methods (which produced the same final models). Models were adjusted to subsets of data including only vaccine period individuals, or excluding vaccinated individuals. Although only 73 of 492 samples were positive for co-colonization (as detailed in the results' section) all 492 samples were used in the multiple logistic regression enabling the analyses of all variables under study. Indeed, according to Peduzzi et al. (Peduzzi *et al.*, 1996), the size of our sample allows models with up to 7-8 predictor variables. Furthermore, according to the later work of Vittinghoff and McCulloch (Vittinghoff & McCulloch, 2007), this number of predictors can safely be double if the aim of the analysis is to detect associations in observational data and not making predictions; or if the high number of predictors is necessary to control possible confounding variables. Our study fitted both conditions.

Permutation analysis was done to test if serotypes were found in co-colonized carriers at frequencies different than expected by chance. For each time period, serotype identifications were randomly allocated to children (including not colonized) 2000 times. Serotype frequencies were maintained. The p-values obtained for all serotypes were corrected for multiple testing by controlling the False Discovery Rate below 0.05 (Benjamini & Hochberg, 1995).

Analyses were performed in SPSS 17.0 and in Matlab 7.7.

Results

Co-colonization detected by colony morphology and by *ply*NCR-RFLP. Of the 492 nasopharyngeal samples studied, two colonies with distinct morphologies were identified in the primary selective agar plate of 32 samples. Among these, two serotypes were isolated in 17 samples; the remaining samples yielded a single serotype. Thus, the co-colonization rate detected by this method was 3.5%. The *ply*NCR-RFLP method detected pneumococcal co-colonization in 20.1% of the 492 samples (n=99), a significantly higher proportion than the one found with the former approach ($p<0.001$, X^2 test).

Confirmation of co-colonization by molecular serotyping microarray. A total of 165 samples were blindly tested using the microarray: the 99 samples in which pneumococcal co-colonization was detected by the *ply*NCR-RFLP, and 66 samples for which there was no evidence of co-colonization. Microarray results were in agreement with those generated by the *ply*NCR-RFLP method.

In addition to identifying the serotypes present in samples, the microarray was also able to detect the presence of non-encapsulated pneumococci and/or closely related *Streptococcus* spp., collectively called non-typeables (NT). These were detected in 40 samples, all of which had an indication of co-colonization by the *ply*NCR-RFLP method. However, as both methods did not confidently discriminate between NT species within co-colonized samples, we decided to exclude all NT from further analysis to avoid possible over-reporting of true pneumococcal co-colonization. Hence, all results presented in this report involve only pneumococci for which serotypes were identified. Still, when a separate analysis was performed, assuming that all NT were *bona fide* non-encapsulated pneumococci, the same conclusions described below were obtained (data not shown).

Upon exclusion of all NTs, the microarray detected more than one pneumococcal serotype (*i.e*., more than one capsulated strain) in 73 samples that had an indication of co-colonization.

Confirmation of serotypes detected by the microarray. All serotypes identified by the microarray were confirmed by PCR using as template purified DNA of the primary selective growth. New primers, targeting specific capsule biosynthetic genes or variants not covered by the CDC scheme, were designed as needed (Supplementary Table 1).

Factors associated with pneumococcal co-colonization. Vaccination status and attendance of day-care center F were the only variables significantly associated with co-colonization and both were protective factors (Table 1). Vaccinated children presented significantly lower (p=0.004; Fisher's exact test) co-colonization rates (8.0%) than non-vaccinated children (regardless of whether the latter were from the pre-PCV7 era (17.3%) or from the PCV7 era (18.3%)) (Figure 1).

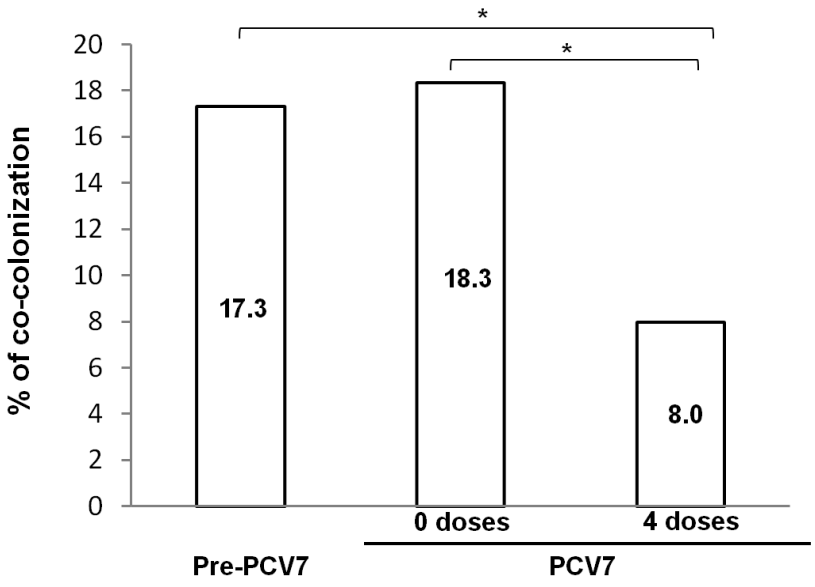

Figure 1. Frequency of co-colonization in the three study groups.

*Statistically significant differences (p=0.004; Fisher's exact test)

Table 1. Risk factors for pneumococcal co-colonization in univariate and multivariate analyses.

Characteristic	No. of carriers	No. of co-colonized carriers (%)	Univariate analysis[c]		Multivariate analysis[d]	
			OR (95% CI)	*p*	OR (95% CI)	*p*
Age (in months)	(-)[f]	(-)[f]	(-)[f]	0.59[e]	0.99 [0.97-1.02]	0.60
Sex						
Male	255	41 (16.1%)	1.23 [0.74-2.02]	0.45	1.18 [0.70-1.99]	0.54
Female	237	32 (13.5%)	Reference		Reference	
Day-care center attended (out of 15)			(-)[f]	0.15[g]		
Unit F[h]	74	4 (5.4%)	**0.29 [0.10-0.82]**	**0.01**	**0.15 [0.04-0.56]**	**0.005**
Other units	418	69 (16.5%)	Reference		Reference	
Time period						
Pre-PCV7	173	30 (17.3%)	0.74 [0.45-1.23]	0.29	1.73 [0.82-3.66]	0.15
PCV7	319	43 (13.5%)	Reference		Reference	
Vaccination status						
Unvaccinated[i]	342	61 (17.8%)	Reference		Reference	
Vaccinated (4 doses)	150	12 (8.0%)	**0.40 [0.21-0.77]**	**0.004**	**0.34 [0.16-0.72]**	**0.005**

[c]Fisher's exact test (Monte Carlo estimation with 10,000 simulations), except when indicated

[d]Multivariate logistic regression

[e]Kolmogorov-Smirnov test

[f]not available for variables that are continuous or have more than two classes

[g]test applied to all day-care centers simultaneously

[h]results are only shown for unit F as the results for other units were not significant

[i]includes unvaccinated children from pre-PCV7 and PCV7 era

Co-colonization patterns. Among the 73 co-colonized samples (excluding NT), the microarray identified two serotypes in 59 samples, three serotypes in 13 samples, and six serotypes in one sample (Table 2). When each type of co-colonization (double, triple, and sextuple) was analyzed separately, a higher proportion of each type of co-colonization was still noted among the unvaccinated children. However, probably due to the low number of observations, the results did not reach statistical significance (Table 2).

Table 2. Colonization events according to study group.

No. of serotypes detected in samples	Pre-PCV7 era, n=173 (%)	PCV7 era		Total, n=492 (%)	*p* value
		Unvaccinated, n=169 (%)	Vaccinated, n=150 (%)		
1	143 (82.6)	138 (81.7)	138 (92.0)	419 (85.2)	0.018[a]
> 1 (all co-colonization samples)	30 (17.3)	31 (18.3)	12 (8.0)	73 (14.8)	0.018[a]
2	25 (14.4)	23 (13.6)	11 (7.3)	59 (12.0)	0.11[a]
3	4 (2.3)	8 (4.7)	1 (0.7)	13 (2.6)	0.08[b]
4	0 (-)	0 (-)	0 (-)	0 (-)	-
5	0 (-)	0 (-)	0 (-)	0 (-)	-
6	1 (0.6)	0 (-)	0 (-)	1 (0.2)	0.65[b]

[a] X^2 test

[b] Fisher's exact test

Serotype distribution. In line with previous observations, a significant serotype replacement effect (of vaccine types (VTs) by non-vaccine types (NVTs)) was observed among samples from the PCV7 era compared to samples from the pre-PCV7 era (Figure 2). This effect was noted both in single and co-colonization events and among vaccinated and non-vaccinated children, although it was more pronounced in the former group.

When the relative proportion of VTs in single and co-colonization in the pre-PCV7 and PCV7 eras were compared, no significant changes were observed (p=0.338, Fisher exact test). However, this was not true for NVTs: in the PCV7 era there were significantly less NVT co-colonization events than would be expected based on the NVT distribution observed in the pre-PCV7 era (p=0.024). This result, suggested that, in the PCV7 era, there was an asymmetric distribution among NVTs found in single or co-colonizing events. Such effect was more pronounced among vaccinated children (where serotype replacement effect was strongest) leading to a decreased rate of co-colonization among this group.

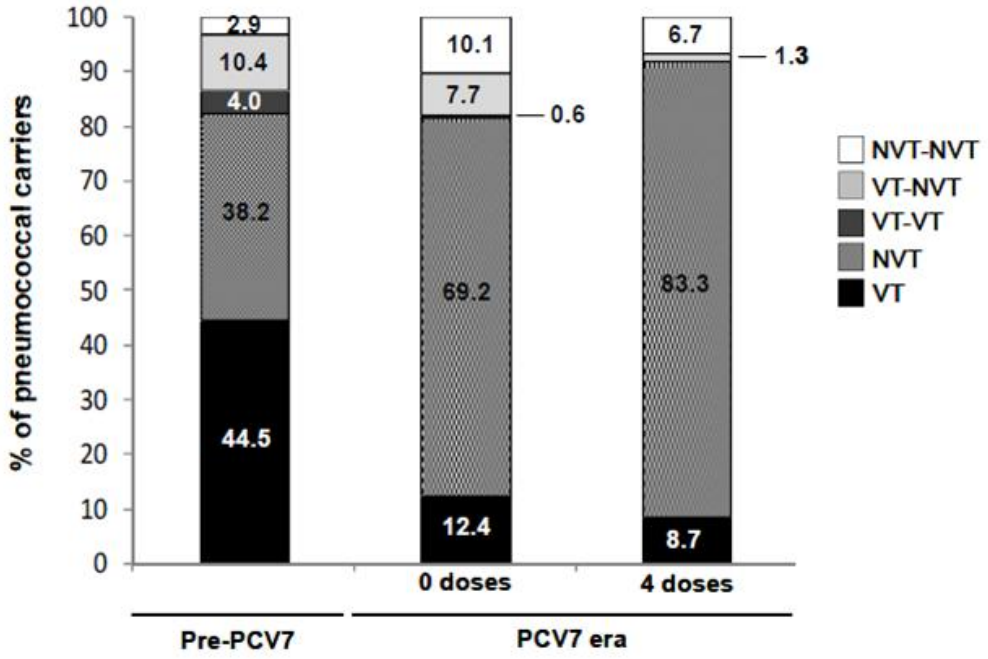

Figure 2. Single and co-colonization events by vaccine-types (VT) and non-vaccine types (NVT) in the three study groups.

Furthermore, the distribution of individual serotypes found in co-colonized samples reflected, in general, the distribution found in samples containing a single strain, i.e., serotypes frequent in single colonization tended to be also frequent in co-colonization (Figure 3). Serotypes 19F and 23F were the most frequent colonizers of the nasopharynx in the pre-PCV7 era and serotypes 6A and 19A dominated in the PCV7 era.

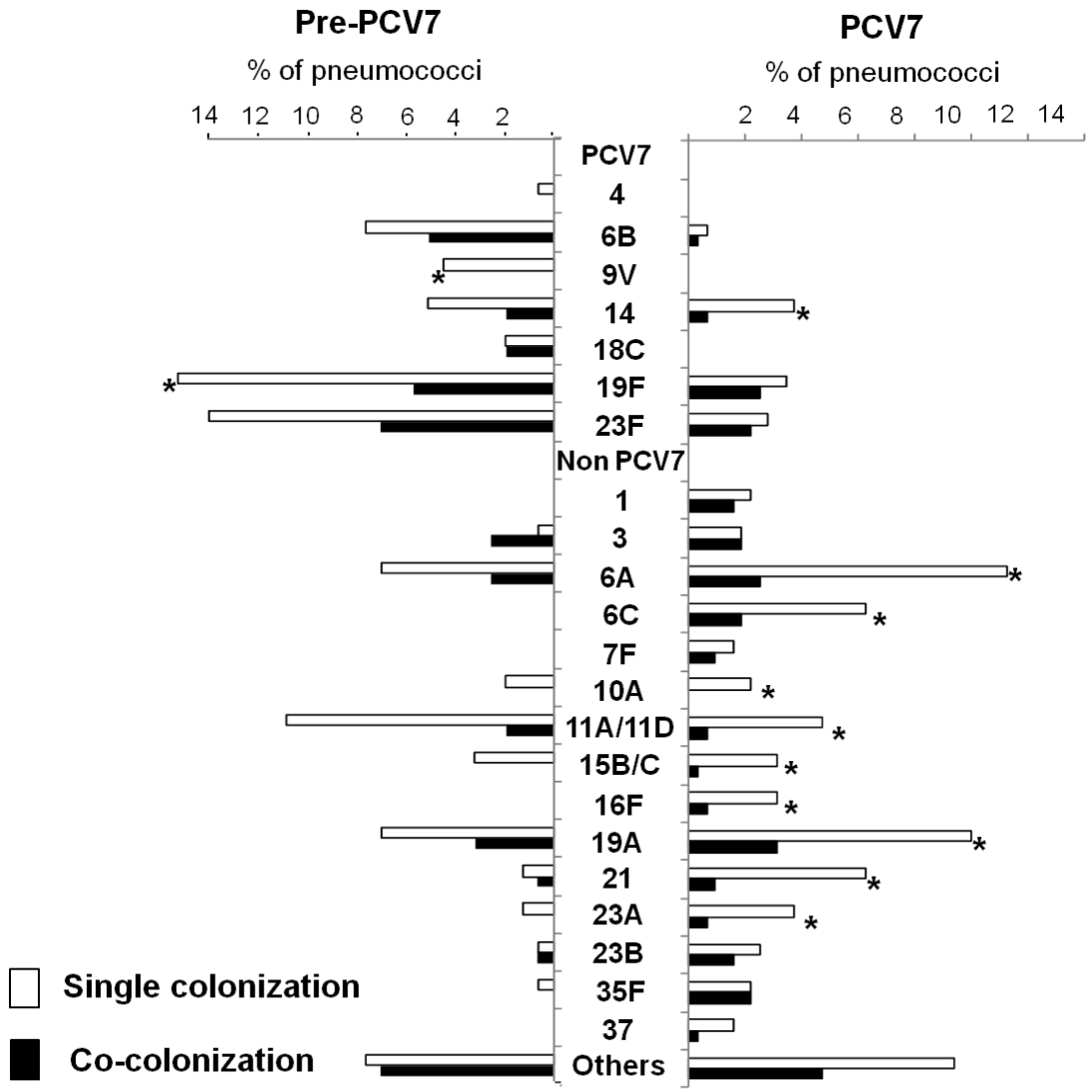

Figure 3. Individual serotype distribution of single and co-colonized samples from the pre-PCV7 and PCV7 eras. Non-vaccine serotypes with an absolute frequency <5 in the PCV7 era were grouped as "others". These include serotypes 8, 9L, 9N, 13, 15A, 17F, 18A, 18F, 19B, 19C, 22F, 24F, 29/35B, 31, 33A/33F, 34, 38, 42, 33B-like, 35A/C, 36/15B-like, and 39-like. *Observed frequency in co-colonization is significantly lower than expected.

Relative abundance of serotypes of co-colonized samples. For each co-colonization event the relative proportion of each serotype was determined by the microarray. We observed that there was a clear dominance of one serotype over the other(s) in a sample in 71.9% the cases involving associations of NVTs. In the associations of two or more VTs, or VTs with NVTs, this dominance was not so evident being 62.5% and 42.4%, respectively.

To investigate if individual serotypes had been detected in co-colonization at a frequency significantly different from the one expected by chance alone, a permutation analysis was done for each time period. Serotypes 9V and 19F (in the pre-PCV7 era), and 6A, 6C, 10A, 11A/11D, 14, 15B/C, 16F, 19A, 21, and 23A (in the PCV7 era) were found less frequently in co-colonization than expected (Figure 3). A more detailed analysis aimed to detect specific associations between pairs of serotypes was not possible due to the low numbers of each pair.

Discussion

We aimed to study the impact of PCV7 on pneumococcal co-colonization rates by combining two recently developed methods, *ply*-NCR RFLP and molecular serotyping microarray, which enabled detection of co-colonization and the identification and relative quantification of the serotypes present in those samples.

After excluding NT species, a co-colonization rate by capsulated pneumococci of approximately 15%, confirmed by both the *ply*NCR-RFLP and microarray methods was observed. The combination of these two methods allowed four times more sensitivity in detection of co-colonization than serotyping of individual colonies exhibiting distinct morphologies.

Our results show that introduction of PCV7 led to a serotype replacement effect among vaccinated children and, although less pronounced, also among non-vaccinated children. As a result, in the PCV7 era, a decrease in the proportion of VTs was observed in single and co-colonized carriers of both groups. These findings are in line with previous observations that indicated that, in settings where vaccination rates are high, vaccination reduces not only the proportion of vaccinated individuals colonized with VTs but also among non-vaccinated

individuals in the community due to an indirect effect resulting from decreased transmission of VTs (Weinberger *et al.*, 2011).

Interestingly, co-colonization rates were significantly lower among vaccinated children (8.0%) than among non-vaccinated children (c.a. 18%, irrespective of whether they were from the pre-PCV7 or PCV7 eras). Of the variables studied, attendance of day-care center F and, more importantly, vaccination status were the only factors that accounted for these phenomena.

Comparison of the characteristics of day-care center F with the other 14 day-care units did not reveal obvious differences between them (in carriage prevalence, area, number of children, crowding, or antimicrobial consumption). However, this center was enriched on serotypes (6A, 11A/D, and 19A) known to be less associated with co-colonization than expected by chance alone (as discussed below) which, per se, could potentially explain this observation.

The finding that co-colonization was diminished among PCV7-vaccinated children is of particular interest as it may have important implications for pneumococcal evolution. Indeed, decreased co-colonization is expected to translate in fewer opportunities for horizontal gene transfer hindering, for example, transference of resistance genes and emergence of vaccine escape recombinants.

But how to explain such observations? A closer inspection of Figure 2 indicates that among vaccinated children a significant reduction of VTs led to an asymmetric redistribution of the proportion of single and multiple NVT carriers. Specifically, it resulted in a higher than expected proportion of single NVT carriers and a lower than expected proportion of NVT/NVT multiple carriers.

One possible interpretation for the latter observation is that some NVTs in circulation in the PCV7 era are more competitive than others, impairing their co-existence in the same ecological niche. Evidence for competition between serotypes in the nasopharynx was

described before and mechanisms leading to it (such as bacteriocin production) have been identified (Claverys & Havarstein, 2007; Lipsitch *et al.*, 2000). Two observations in the study support this hypothesis: (i) the fact that in the association of two or more NVTs, there was a clear dominance of one serotype in 71% of the cases, while such high values were not observed in the association of two or more VTs (63%) or in the association of VTs with NVTs (42%); and (ii) in the PCV7 era, there were a number of NVTs that were found less frequently in co-colonization than would be predicted by chance alone. These serotypes were among the group of the most prevalent ones in single colonization for the same period suggesting they are highly competitive.

Our results contrast with those from a recent study in Switzerland that reported a stable rate of co-colonization upon introduction of PCV7 and a balanced co-existence of serotypes found in co-colonized samples (Brugger *et al.*, 2010). That study, however, differs from ours in several aspects hindering comparisons between findings. The study population consisted of patients with acute otitis media or pneumonia (and hence, not healthy), children and adults were included, colonization rates were significantly lower (even when the same age groups were compared), and the starting material was the swab without a culturing step. Furthermore, if competition between serotypes is indeed relevant, local serotype distribution may further affect the results obtained. Additional studies are warranted to explore this subject.

Of note, we observed that in our setting (as in many others), in the vaccine era, serotypes 6A and 19A were the most frequent colonizers of the nasopharynx in single and co-colonization. As the recently introduced PCV13 includes these two serotypes, it will be important to monitor how co-colonization is affected by it.

Our study has some limitations. Firstly, it is a cross-sectional study and therefore duration of carriage, known to vary according to the serotype, was not taken into account (Gray *et al.*, 1980). This has at least two implications: on one hand, the co-colonization rate may have

been underestimated as serotypes with shorter duration of carriage may have been missed; on the other hand, we were unable to determine whether the co-colonized samples result from a truly co-existence colonization event or merely reflect a transitional state between serotypes colonizing the nasopharynx.

Secondly, the direct culture of the nasopharyngeal swab has some disadvantages. As with any culturing step, the chance of occurrence of sample contamination during handling increases. In addition, variation in the inoculum size may have occurred. This could have been partially overcome if the swab would have been first diluted in a liquid medium and, afterwards, a fixed amount of the liquid sample plated. Also, the use of a cultivation step and the choice of a selective (gentamycin blood agar) medium may have altered the composition of the sample. Finally, the culturing step may lead to false-negative results (as we analyzed viable cells only). Still, despite these limitations, our strategy has the advantage of increasing the amount of pneumococcal DNA in the samples. Direct analysis of the swabs often leads to limited amounts of DNA hindering further processing of the sample; for example in a previous study using the *ply*NCR-RFLP method, 21% of the samples did not yield sufficient DNA for analysis (Brugger *et al.*, 2009).

The third limitation is the current inability of the microarray to discriminate between non-encapsulated pneumococci from other closely related *Streptococcus* spp. such as *S. mitis* when within a complex mixture containing other pneumococci. In this study, we ignored the presence of these NT organisms but a separate analysis taking into account these results mimicked the general observations reported here. However, co-colonization with non-encapsulated pneumococcus or closely related species is of interest as this represents the wider gene pool available for horizontal gene transfer.

Finally, the inclusion of older children (up to 71 months) might be considered a limitation as it is not clear whether conjugate vaccines administered in the first and second year of life will have an effect on colonization 3-4 years afterwards. Still, a previous study by Millar *et al.* on

the long-term effect of PCV in a community using a 3+1 schedule, found a significantly lower prevalence of VT carriage 27 months after vaccination when compared with control communities (Millar *et al.*, 2006), suggesting there might be a prolonged effect of PCV7 on colonization. Furthermore, if we consider the opposite scenario, that is, that children of older ages are no longer protected by PCV7 then we would expect this group to have higher carriage of VTs, which would lead to a net increase in VT among the vaccinated group. This, in turn, according to our data, would result in a global increase in co-colonization among this group. In other words, the results presented by us for the vaccinated group would have a bias towards an increasing co-colonization rate. Still, the rate observed was significantly lower than among non-vaccinated children supporting our observations and suggesting that, if anything, the exclusion of older children would result in a even stronger disparity between co-colonization rates among vaccinated and unvaccinated children.

Our study has also some strength. The collection of samples used was well defined and matched and the methods used have high sensitivity. This allowed us to take some important conclusions in the context of co-colonization. Finally, we are convinced that, to our knowledge, this is one of the very few studies to date focusing on the impact of PCV7 on pneumococcal multiple carriage and is the first to report a significant difference between co-colonization rates of vaccinated and non-vaccinated children.

In summary, this study suggests that PCV7-vaccinated children have lower rates of pneumococcal co-colonization, resulting in decreased opportunities for horizontal gene transfer between strains. This represents a novel potential benefit of multivalent pneumococcal conjugate vaccines. Considering that these results may depend on local epidemiological factors and that serotype redistribution is occurring as novel vaccines are being introduced, additional studies are warranted to verify if similar results are obtained.

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Supplementary material

Table S1. List of oligonucleotides designed for this study.

Primer name	Serotype targeted	Gene	Fragment size (bp)	Primer sequence (5'-3')
wchL_15B_f	15B	*wchL*	449	5'-GTGATTTTGGTGAATGATGGGTCTAACGATTCT-3'
wchL_15B_r				5'-GACATATCTTCACCTAGTGACATCTCAGTCG-3'
wcxR_16A_f	16A	*wcxR*	387	5'-GCCCCTAGAAAAGTTCCTGCTAGCATCACG-3'
wcxR_16A_r				5'-GTGGATAGGGGACAACGATAG-3'
wzy_19B/C_f	19B/C	*wzy*	375	5'-CCAAGTAAATGCTCTAATTCCGAGAG-3'
wzy_19B/C_r				5'-GCATTCGTTTATGGAGGTGGATTGG-3'
wchU_19C_f	19C	*wchU*	371	5'-GCCCCCACATAATGAACTTTTTCAACTGG-3'
wchU_19C_r				5'-GCTCTTTGCTATGGTTTACCATGTATCGG-3'
wzy_27_f	27	*wzy*	617	5'-GCTCTCCCATGCATGCTTGCAGGATTTAGAGC-3'
wzy_27_r				5'-GACACTACTCCTAAGGAAAAGAGGGAGGCTATACC-3'
wciO_33B_f	33B	*wcjO*	245	5'-GGTAGACCTCTATCATACATATTGACAATTCC-3'
wciO_33B_r				5'-GCATCTATAACACTTCCCATAGGAGAGGTAATTCCC-3'
wcjH_35F_f	35F	*wcjH*	295	5'-CAGCATCTTTATACATGCTCCG-3'
wcjH_35F_r				5'-CTGTGAAACCTGGTTTGC-3'
wcjA_36_f	36	*wcjA*	622	5'-GGTGGCGCTGAGAGGATTGTGTACCAG-3'
wcjA_36_r				5'-CAGTTCTAAGCTCTCCATCACCAAC-3'
wcrD _39_f	39	*wcrD*	562	5'-GGTGGACATGGGTCAACGATTAATGTTGGT-3'
wcrD _39_r				5'-AGTATGTCAATACGTCTCTGACTACTGG-3'

Table S2. Age distribution in the three groups.

Age (yr)	No. of children per group (%)		
	Pre-PCV7 period	PCV7 period (0 doses)	PCV7 period (4 doses)
<2	15 (8.7)	4 (2.4)	11 (7.3)
2-<4	50 (28.9)	58 (34.3)	63 (42.0)
4-<6	108 (62.4)	107 (63.3)	76 (50.7)
Total:	173	169	150

Comparison of age distribution in the three groups was done by a Kolmogorov-Smirnov test using the age of each child in months. The results were not significantly different (p=0.614).

Chapter III

Impact of the 13-valent pneumococcal conjugate vaccine on *Streptococcus pneumoniae* multiple serotype carriage

Submitted:

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Contributions:

C. Valente was responsible for all experimental work with the exception of the comparative genomic hybridization experiments, which were performed by K. Gould and analyzed by J. Hinds.

Summary

Introduction: Pneumococcal multiple serotype carriage is important for evolution of the species and to understand how the pneumococcal population is changing with vaccination. We aimed to determine the impact of the 13-valent pneumococcal conjugate vaccine (PCV13) on multiple serotype carriage.

Methods and Materials: Nasopharyngeal samples from fully vaccinated pneumococcal carriers (4 doses of PCV13, n=141, aged 18-72 months) or from non-vaccinated pneumococcal carriers (0 doses of any PCV, n=140, same age group) were analyzed. Multiple serotype carriage was evaluated by DNA hybridization with a molecular serotyping microarray that detects all known serotypes.

Results: Vaccinated children had a lower prevalence of multiple serotype carriage than the non-vaccinated group (20.6% vs 29.3%, p=0.097), and a significantly lower proportion of PCV13 serotypes (6.4% vs 38.5%, p=0.0001). PCV13 serotypes found among vaccinated children were mostly detected as a minor serotype in co-colonization with a more abundant non-vaccine serotype. Vaccinated children were colonized by a significantly higher proportion of commensal non-pneumococcal *Streptococcus spp*. (58.2% vs 42.8%, p=0.012). In vaccinated children there were significantly less non-vaccine type (NVT) co-colonization events than expected based on the distribution of these serotypes in non-vaccinated children.

Conclusions: The results suggest that vaccinated children have lower pneumococcal multiple serotype carriage prevalence due to higher competitive abilities of non-vaccine serotypes expanding after PCV13 use. This might represent an additional benefit of PCV13, as decreased co-colonization rates translate into decreased opportunities for horizontal gene transfer and might have implications for the evolution and virulence of pneumococci.

Introduction

Streptococcus pneumoniae is an important cause of infectious disease, with a high rate of mortality worldwide, particularly among young children, the elderly and the immunocompromised (O'Brien *et al.*, 2009).

Despite the high burden, invasive pneumococcal disease is incidental (Bogaert *et al.*, 2004 ; CDC, 2012). Nasopharyngeal colonization is the natural lifestyle for the pneumococcus, with particularly high prevalence among young children (Gray *et al.*, 1980). Colonization is key in pneumococcal biology as it precedes disease, facilitates transmission between hosts for perpetuation of the species; and allows sustained evolution of the species to take place.

The pneumococcus evolves mainly by recombination through horizontal gene transfer (HGT) occurring when multiple strains or serotypes of pneumococci coexist, a phenomenon also known as co-colonization. Likewise, HGT with closely related co-colonizing commensals such as *S. mitis* and *S. pseudopneumoniae* can also occur (Kilian *et al.*, 2008).

Co-colonization or multiple serotype carriage is frequent. Recent studies reported multiple serotype carriage prevalence of up to 40% in children (Kamng'ona *et al.*, 2015; Kandasamy *et al.*, 2015; Valente *et al.*, 2012). An accurate detection of multiple serotype carriage in surveillance studies is relevant (i) for the understanding of the intra-species interactions, (ii) to obtain a comprehensive knowledge of how the pneumococcal population is being altered by anti-pneumococcal vaccination, and (iii) to predict the vaccine impact on disease when using models based on carriage prevalence (Brueggemann *et al.*, 2003; Sá-Leão *et al.*, 2011; van Hoek *et al.*, 2014).

In recent years, with the increasing availability of highly sensitive serotyping methods that are able to detect multiple serotype carriage, reports on co-colonization are

becoming more frequent (Brugger *et al.*, 2010; Kamng'ona *et al.*, 2015; Kandasamy *et al.*, 2015; Turner *et al.*, 2011; Valente *et al.*, 2012). In a previous study conducted to determine the prevalence of multiple serotype carriage among Portuguese children and the impact of vaccination with the 7-valent pneumococcal conjugate vaccine (PCV7), we demonstrated that PCV7 vaccinated children were significantly less co-colonized than non-vaccinated children due to an uneven distribution of serotypes selected by PCV7 in single and co-colonization events (Valente *et al.*, 2012).

The 13-valent pneumococcal conjugate vaccine (PCV13) became commercially available in Portugal in January 2010 replacing PCV7. Although at the time of this study none were introduced in the National Immunization Program (NIP), these vaccines are highly prescribed and their usage has been recommended by the Portuguese Pediatric Society . For this reason, vaccine coverage has been high, reaching 63% by 2012 (data from the National Statistics Institute (INE) and IMS). Very recently, in June 2015, PCV13 was introduced in the NIP on a scheme of two doses followed by a booster dose.

The impact of PCV13 on carriage is being studied in several countries and there are already some studies addressing this issue (Chang *et al.*, 2015; Gladstone *et al.*, 2015; Steens *et al.*, 2015). However, to our best knowledge the impact of this vaccine on carriage of multiple serotypes and co-colonization with other *Streptococcus spp.* has not been addressed.

The aims of this study were to determine the impact of PCV13 on pneumococcal multiple serotype carriage and to compare the results with those obtained for PCV7.

Materials and methods

Study design. Nasopharyngeal (NP) swabs collected from healthy children attending

day-care centers in Oeiras and Montemor-o-Novo, Portugal, were retrospectively selected. Samples were collected in the winter months of January to March between 2011 and 2015. The following criteria were used for selection of samples to be analyzed in this study: (i) swabs were obtained from children aged 18-71 months; (ii) children had not received antibiotic within the month preceding sampling; and (iii) swabs yielded a pneumococcal positive culture.

Samples were clustered into two groups: one group included samples collected from children who had not been vaccinated with any PCV (n=150); the other group included samples collected from fully PCV13-vaccinated children, *i.e.*, children who had received 4 vaccine doses (n=150).

Ethics statement. This study was approved by the Ethics Research Committee of the NOVA Medical School/Faculdade de Ciências Médicas - New University of Lisbon (CEFCM) (47/2014/CEFCM). Samples were collected upon signed informed consent from the parents or guardians of participating children. All information was processed anonymously.

Sample collection and isolation of pneumococci. NP samples were collected by pediatric nurses. In 2011 and 2012 mini-tip calcium alginate sterile swabs were used and inoculated directly within 4 hours in a primary selective plate of 5% blood trypticase soy agar containing gentamicin (5 mg/liter) to select for *S. pneumoniae*. Samples from 2014 and 2015 were collected and isolated according to the standard procedures recommended by the WHO (Satzke *et al.*, 2014). Swabs, the total bacterial lawn of the primary gentamicin blood plate and pneumococcal isolates were frozen at -80ºC in 1ml of STGG medium containing 30% glycerol.

DNA isolation. STGG tubes containing the nasopharyngeal swab were thawed on ice and vortexed. 50 µl aliquots were plated onto tryptic soy blood plates supplemented with gentamicin using a spreader and incubated overnight at 37ºC in 5% CO_2. On the

following day, a plate sweep of the total bacterial growth was collected and DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen, Germany).

Detection of multiple serotype carriage by microarray. The BµG@S SP-CPSv1.5.0 *S. pneumoniae* molecular serotyping microarray was used following standard protocols previously described (Brugger *et al.*, 2010; Turner *et al.*, 2011). Genomic DNA ULS labeling and hybridization protocols were used and microarray slides were scanned using a high-resolution microarray scanner (Agilent Technologies, USA). Microarray data was analyzed using a Bayesian hierarchical model to determine the serotype, or combination of serotypes, present in the sample and to assign their relative abundance (Newton *et al.*, 2011). For the interpretation of the microarray quantification results, a serotype was classified according to its relative abundance in the sample as: dominant if ≥70%; co-dominant if >30% and <70% and minor if ≤30%.

Statistical analysis. Statistical significance was accessed through Fisher's exact tests. For all analyses differences were considered statistically significant when $p<0.05$.

Results

Microarray serotyping and detection of co-colonization. The microarray analysis detected *S. pneumoniae* DNA in a total of 281 samples – 140 samples collected from non-vaccinated children and 141 samples collected from vaccinated children. Samples in which the microarray failed to detect pneumococcal DNA were excluded from the study (n=19). In four of these samples one encapsulated pneumococcal strain had been isolated before, but probably due to low abundance and sampling, it was not detected when the DNA of the primary culture was probed in the microarray. In the remaining 15 samples, putative non-encapsulated pneumococci were isolated and were later confirmed to be strains of other closely related *Streptococcus spp.*

Among the 281 samples containing pneumococci, the microarray identified 345 pneumococcal strains of known serotypes, 30 pneumococcal strains of non-encapsulated lineages (herein called non-typeable or NT) and 302 *Streptococcus spp*. strains. The pneumococcal population was distributed throughout 40 serotypes and NT strains (Figure 1). Pneumococcal co-colonization was detected in 25% of the samples (70 out of 281 samples). Half of the samples (50.5%) contained at least one *Streptococcus spp*. strain in addition to *S. pneumoniae*.

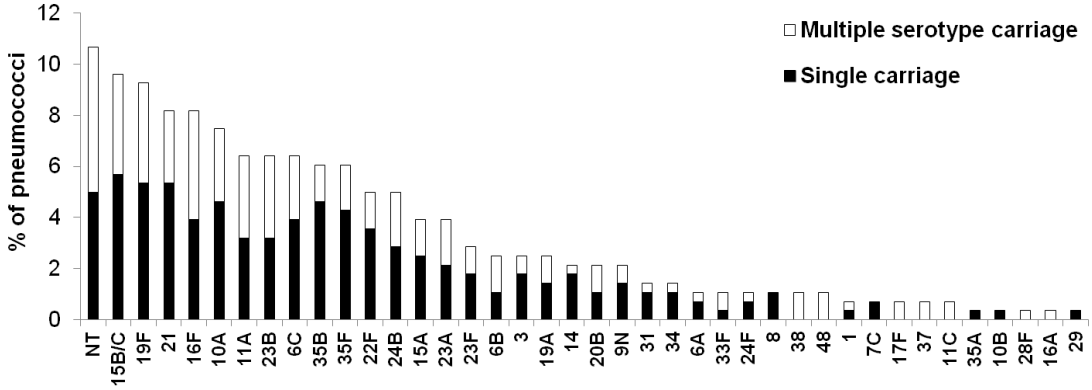

Figure 1. Serotype ranking in the study population obtained through the detection of single and co-colonization events.

The microarray identified three types of non-typeable non-encapsulated pneumococci that are genetically distinct: NT2, NT3b and NT4b detected in 1.1%, 7.5%, and 2.1% of the samples, respectively (Park *et al.*, 2012; Salter *et al.*, 2012). For subsequent analysis we have grouped non-encapsulated strains into a single group (NT), which was collectively the most prevalent pneumococcal type.

The most abundant *Streptococcus* species (other than pneumococci) identified were *S. mitis*, *S. oralis*, *S. infantis* and *S. salivarius* present in 46.3%, 34.9%, 32.7% and 30% of the samples, respectively. Other species identified included *S. pseudopneumoniae*, *S. sanguinis*, *S. constellatus*, *S. anginosus* and *S. intermedius*, present in up to 5% of the samples. Among these samples, in some cases the microarray detected the presence of a few capsule biosynthesis gene homologues, but never the full complement of *cps* genes expected for any known pneumococcal serotypes.

In general, serotypes found in co-colonization had comparable frequencies as minor or non-minor serotypes. The exception to this scenario were NT pneumococci, which were found as minor serotype in the majority of co-colonization events (p=0.0164, Fisher's exact test) (Figure S1). Still, the ranking frequencies of several serotypes would change if only the dominant serotype in the samples had been detected (Figure S1).

Pneumococcal co-colonization in PCV13 vaccinated and non-vaccinated carriers. The prevalence of pneumococcal multiple serotype carriage among PCV13 vaccinated carriers was lower (20.6%) than among the non-vaccinated group (29.3%), although the difference was not statistically significant (p=0.0973, Fisher's exact test). In both groups most multiple serotype carriage samples contained two serotypes and a maximum of four serotypes were detected in 5 samples. In multiple serotype carriage samples, a clear dominance of one serotype was noted in most cases with no significant differences between the two groups (Table 1).

Table 1. Comparison between nasopharyngeal samples obtained from PCV13 vaccinated and non-vaccinated pneumococcal carriers

Characteristic	PCV13 vaccinated (total=141) n, (%)	Non-vaccinated (total=140) n, (%)	p-value[a]
Multiple serotype carriage	29 (20.6%)	41 (29.3%)	0.0973
No. serotypes detected			
Two	20 (69.0%)	32 (78.1%)	0.4182
Three	8 (27.6%)	6 (14.6%)	0.2305
Four	1 (3.4%)	3 (7.3%)	0.6369
Relative quantification			0.1560
Dominance of one strain	19 (65.5%)	34 (82.9%)	
Co-dominance	10 (34.5%)	7 (17.1%)	

[a] Fisher's exact test.

Overall carriage of PCV13 vaccine types was significantly lower in the vaccinated group, compared to the non-vaccinated group (6.4% vs 38.5%, p=0.0001, Fisher's exact test). However, when single and multiple serotype carriage events were analyzed separately, the trend was maintained but only reached statistical significance in single carriage events (2.1% (3/141) vs. 26.4% (37/140), p<0.0001 in single carriage and 4.3% (6/141) vs. 12.1% (17/140), p=0.21 in multiple carriage) (Figure 2).

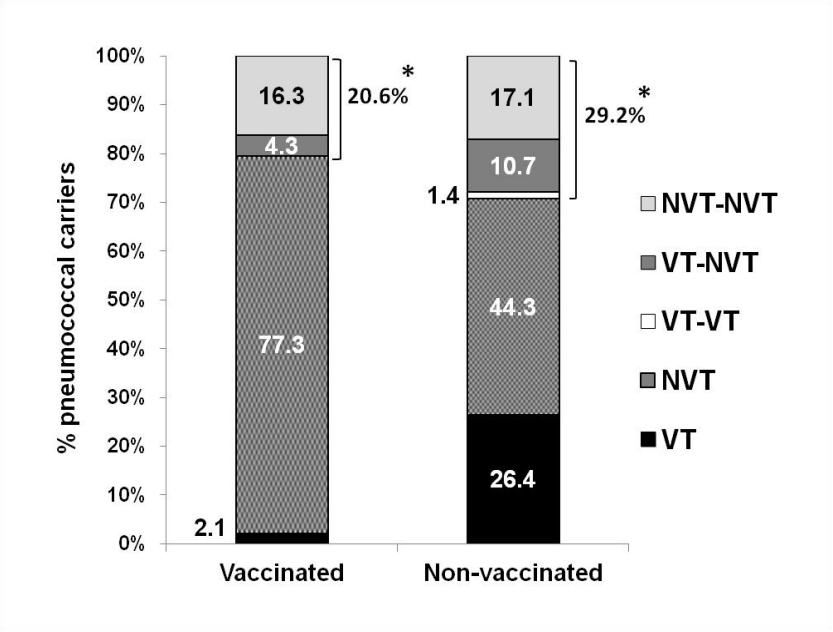

Figure 2. Single and co-colonization events by PCV13 vaccine types (VT) and non-PCV13-vaccine types (NVT) in PCV13 vaccinated and non-vaccinated children. Asterisks indicate total prevalence of multiple serotype carriage.

Among the few (n=9) vaccinated children that carried PCV13 serotypes, six had them in co-colonization with a non-vaccine type (NVT); in five of these events the vaccine type was in minority. By contrast among the 54 non-vaccinated children that carried PCV13 serotypes, in the majority (37, 69%) it was found as a single serotype. In the remaining carriers, PCV13 serotypes were found in co-colonization and were dominant in the majority of the events (11 of 17) (Figure 3).

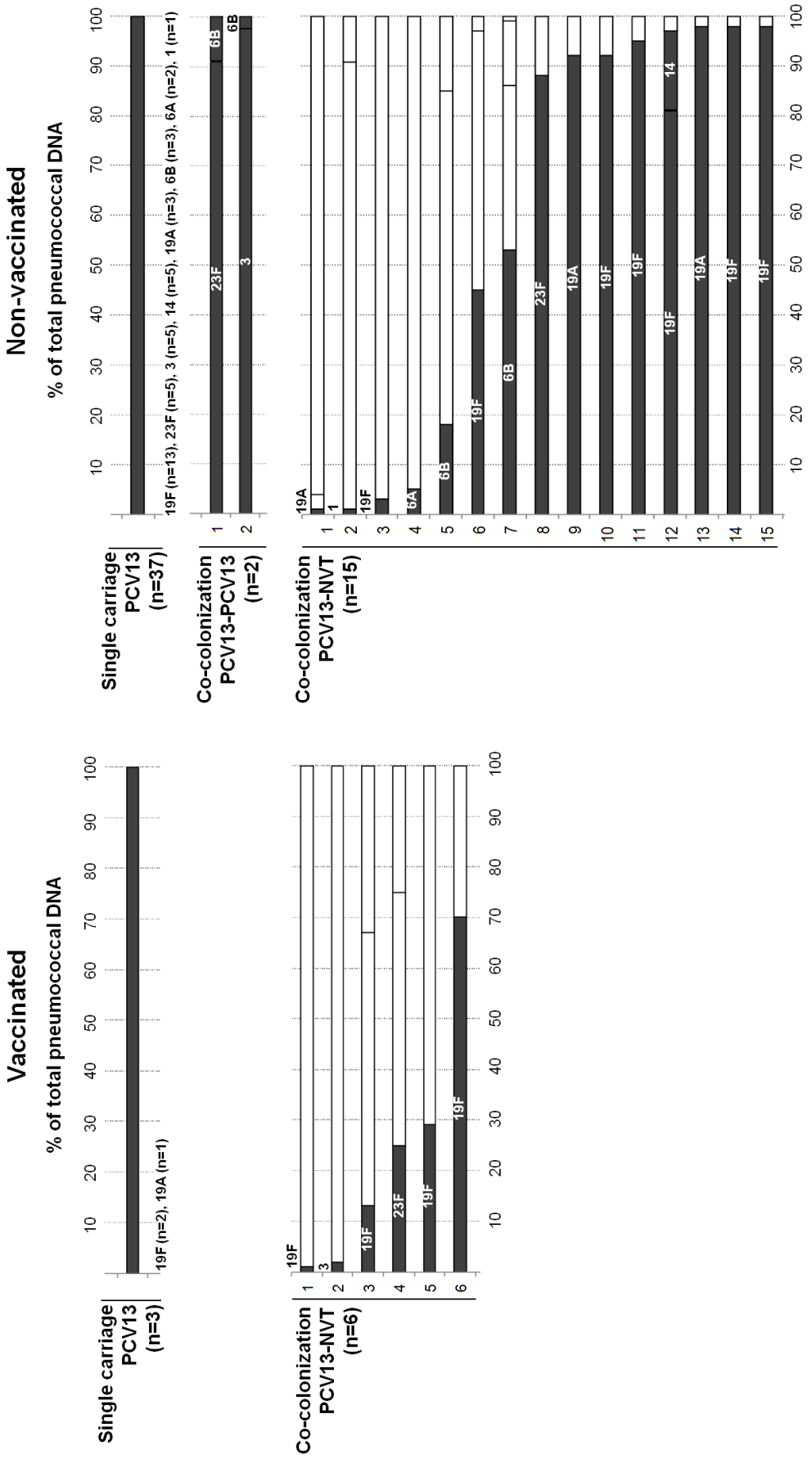

Figure 3. Carriage and relative abundance of PCV13 serotypes in non-vaccinated and vaccinated children, in single and co-colonization events. Bars correspond to pneumococcal samples. Black, PCV13 serotype; white, non-PCV13 serotype (NVT). Co-colonization events are divided in PCV13-PCV13 and PCV13-NVT, according to types of serotype associations.

Of note, serotype 19F accounted for 40% (n=26 out of 66) of all PCV13 strains identified by the microarray and was the most prevalent PCV13 serotype in vaccinated and non-vaccinated children, both in single and co-colonization events (Figure 3).

Of all types, NT strains were the most prevalent pneumococcal type identified in single and multiple carriage events, being present in 11.4% of co-colonized samples. Apart from type NT3b which was found as a dominant strain in three multiple carriage events, all non-encapsulated strains were found co-colonizing as a minor serotype.

Serotype distribution. Comparison of the two groups of carriers concerning the relative fraction of VTs occurring in single and multiple serotype carriage events revealed no differences between vaccinated and non-vaccinated children (26.4/12.1 vs 2.1/4.3, p=0.0626, Fisher's exact test, Figure 2). In contrast, the same comparison for NVTs showed that the relative fraction of these serotypes occurring in single and co-colonization events was different among the two groups: 77.3/20.6 in vaccinated vs 44.3/27.8 in non-vaccinated carriers (p=0.036, Fisher's exact test) (Figure 2).

Associations in co-colonization. We investigated associations among the co-colonized samples. As the number of serotypes present in co-colonization was high (n=38), the frequency in which specific pairs of serotypes were found together was low. Vaccinated children were co-colonized by a lower number of serotypes (n=26) compared to the non-vaccinated group (n=35), which translated in a less complex network of dual interactions (Figure 4).

Among vaccinated co-colonized children the most frequent pneumococcal types were NT strains and serotypes 16F, 11A 15B/C and 21. Among non-vaccinated co-colonized children the most frequent types were NT, 19F, 23B, 6C, 15B/C, and 16F.

Carriage prevalence of other *Streptococcus spp*. was high in both groups but significantly higher in vaccinated children, compared to the non-vaccinated group (58.2% vs 42.8%, p=0.0122, Fisher's exact test) (Figure 4).

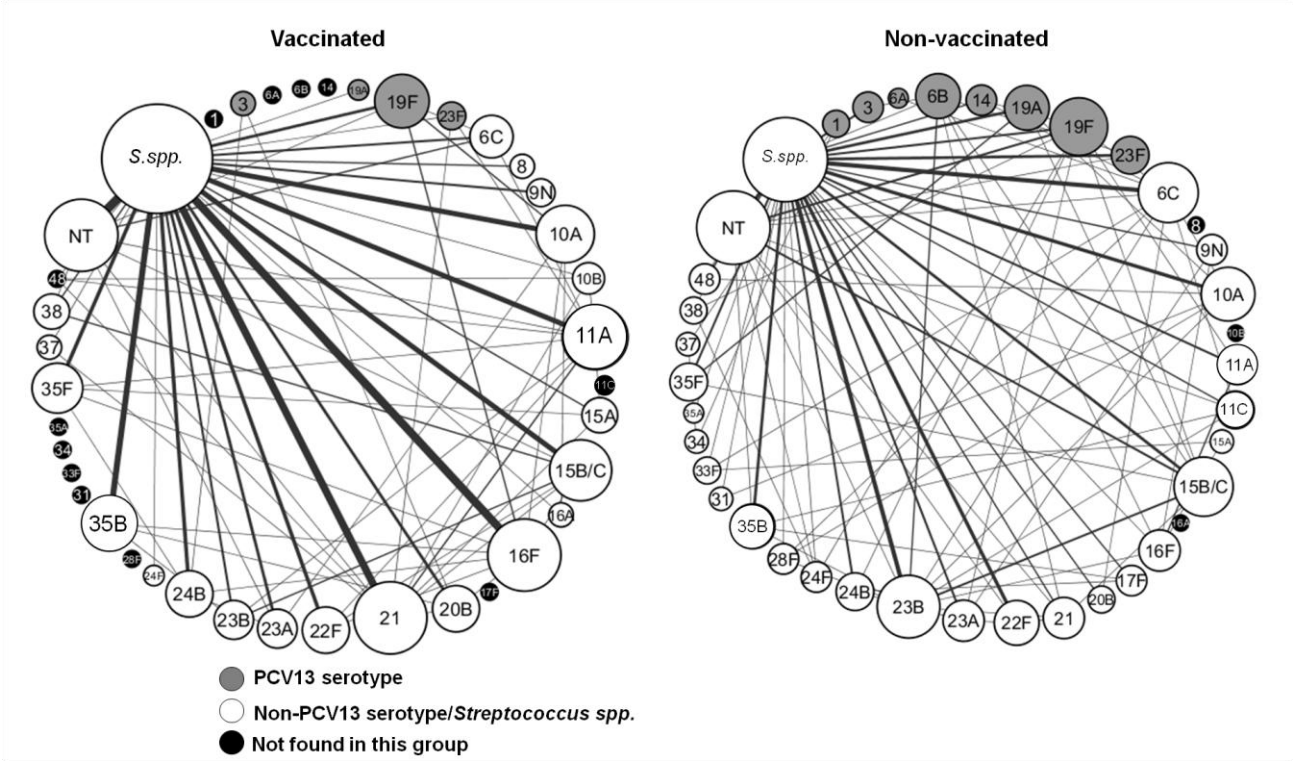

Figure 4. Node plot of strains co-colonizing non-vaccinated and vaccinated children. The size of each node is proportional to the number of interactions in which each serotype is found. The width of the edges is proportional to the frequency in which two serotypes are found together.

The most frequent association detected was between non-typeable pneumococci and non-pneumococcal *Streptococcus spp*. Other frequent associations of *S. pneumoniae* serotypes were 19F-NT, 15B/C-NT, 6C-NT (Figure 4).

Discussion

We have used a molecular serotyping microarray to evaluate the impact of PCV13 on pneumococcal multiple serotype carriage, through the comparison of a group of children who had received four PCV13 doses with another group that had not been vaccinated with any PCV. This method was recently described as the top-performing method currently available to study multi-serotype carriage (Satzke *et al.*, 2015).

The results show that vaccinated children had lower multiple serotype carriage prevalence, compared to the non-vaccinated group (29.3% vs 20.6%), although the difference between groups was not statistically significant.

As expected, we observed that use of PCV13 in Portugal has led to serotype replacement in vaccinated children, a result that is in line with those observed in other countries (Moore *et al.*, 2015; Waight *et al.*, 2015). The use of the microarray has shown that vaccinated children not only carry less VTs but also tend to have them as a minor serotype in multiple carriage with a NVT. On the other hand, among non-vaccinated children carriage of VTs is frequent and often found in single carriage events. When in multiple carriage VTs tend to be the dominant type in this naïve group.

Moreover, we observed that vaccinated children were colonized by a lower number of serotypes and had a higher prevalence of NT and *Streptococcus spp.*, compared to the non-vaccinated group, suggesting niche replacement by less virulent strains. This observation suggests that PCVs, by eliminating highly competitive serotypes from the nasopharyngeal niche, are allowing the expansion of commensal streptococci. This hypothesis is further supported by a study in which the nasopharyngeal microbiota profiles of vaccinated and non-vaccinated children were compared, showing expansion of other *Streptococcus spp.* and other commensals in vaccinated children (Biesbroek *et al.*, 2014).

Yet another difference observed between vaccinated and non-vaccinated children was the asymmetric distribution of NVTs in single and multiple carriage events when the two groups were compared. In vaccinated children the proportion of multiple carriage events with NVTs was lower than it would be expected by chance alone, based on the prevalence of these types in the non-vaccinated group. This observation supports that pneumococcal serotypes have different competitive abilities (Lipsitch *et al.*, 2000; Trzcinski *et al.*, 2015; Valente *et al.*, 2012) and that some NVTs that became more

prevalent following PCV13 introduction might be highly competitive, preventing their co-existence with other pneumococci. This hypothesis might also explain why vaccinated children, where serotype replacement is more pronounced, present lower co-colonization prevalence.

The results obtained in this study are in agreement with a previous study with a similar aim conducted in the PCV7 era (Valente *et al.*, 2012). In both studies we found lower co-colonization rates among vaccinated children and an asymmetric distribution of NVTs in single and multiple carriage events in this group. Results from both studies seem to suggest that PCVs might have the additional benefit of decreasing pneumococcal co-colonization events, and thus, the opportunities for horizontal gene transfer in pneumococci (Hiller *et al.*, 2010). In addition, it is possible that our observations reflect a transient phenomenon of early vaccine effect before equilibrium on serotype distribution has been reached.

Our study has some limitations. First, there were differences in the methodology for isolation of *S. pneumoniae* in the years of 2011/2012, compared to 2014/2015 that could potentially interfere with results. To rule out this possibility, all analyses were repeated excluding samples from 2011 and 2012 and the results obtained mimicked the ones reported in this study, apart from the difference in carriage of *Streptococcus spp.* between vaccinated and non-vaccinated children, which became non-significant but still showed the same trend. Second, the culturing step of the nasopharyngeal swab could potentially have altered the composition of the sample and, thus, interfere with the detection and relative quantification of pneumococci, or induce a bias towards the detection of viable cells only, resulting in false-negative results. Nevertheless, we are convinced that the increase in the total pneumococcal DNA induced by the culturing step is an advantage that surmounts the referred limitations, as direct analysis of swab DNA results in a lower sensitivity of the microarray for detection of the low abundant minor serotypes.

Our study has also some strength. To our knowledge, this is the first study focusing on the impact of PCV13 on pneumococcal co-colonization, reporting differences between vaccinated and non-vaccinated children that might represent novel benefits of PCVs. Also, the use of a highly sensitive microarray allowed us to detect minor serotypes that would otherwise be missed, and to assess how the pneumococcal population structure might be altered by the detection of those serotypes, namely by the unmasking of some serotypes that might be more prevalent than initially thought. In addition, because the microarray incorporates all capsule biosynthesis genes, plus reporters for the *S. pneumoniae* genome backbone and species discrimination, the use of this methodology allowed us to correctly identify pneumococcal strains and to report on the prevalence of closely related *Streptococcus spp*.

A word of caution regarding our study is the fact that we cannot exclude the hypothesis that the observed effect of PCV13 on co-colonization might by restricted to the population studied here. Although we have now addressed this effect of PCVs on co-colonization twice with concordant results (Valente *et al.*, 2012), the observed effects might be a consequence of the serotype distribution in our setting. Additional studies would be of interest to ascertain whether this is the case.

In summary, this study corroborates our previous observations regarding the impact of conjugate vaccines on pneumococcal co-colonization, showing that vaccinated children have lower multiple serotype carriage rates than non-vaccinated children. This observation has implications for the virulence and evolution of pneumococci, as it might result in decreased opportunities for horizontal gene transfer between strains. Of note, we show a very high prevalence of non-encapsulated pneumococci and *Streptococcus spp*., particularly among vaccinated children. The role of these species in the nasopharyngeal ecosystem and in the evolution of pneumococci are questions that remain to be addressed but overall our findings suggest a niche evolution towards commensality.

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Supplementary material

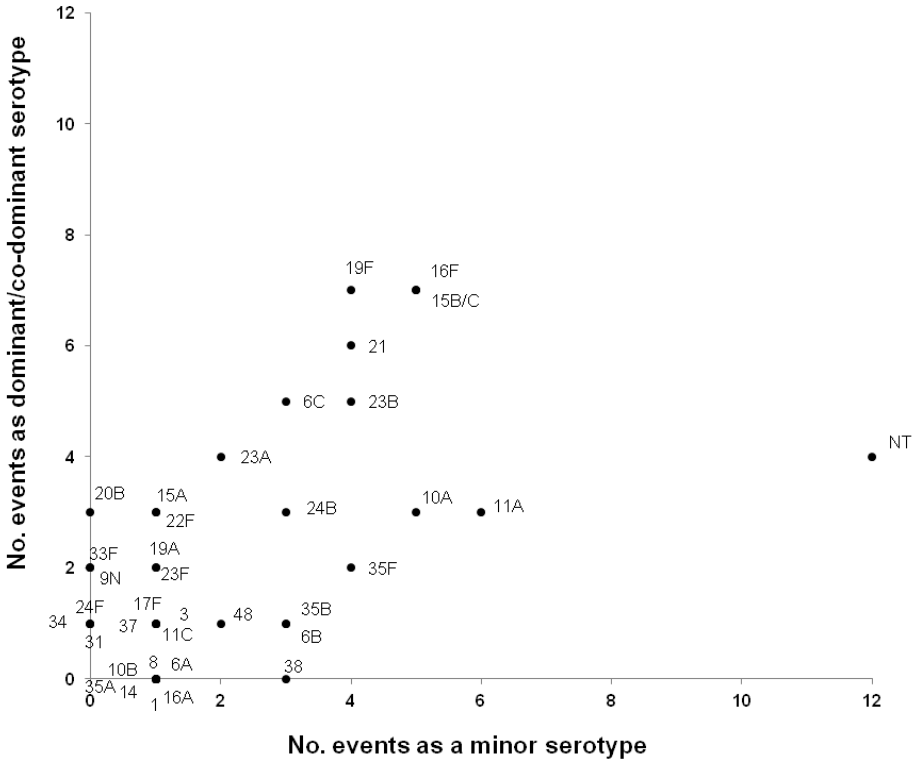

Figure S1. Frequency of serotype occurrences as minor or dominant/co-dominant strain in multiple serotype carriage events. Minor serotype defined as having a relative abundance ≤30% of total pneumococcal DNA; Dominant/co-dominant serotype defined as having a relative abundance >30% of total pneumococcal DNA.

Chapter IV

Selection of distinctive colony morphologies for detection of multiple carriage of *Streptococcus pneumoniae*

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Contributions:

C. Valente performed all the analyses. This study was nested in the studies presented in Chapters II and III.

To the Editors:

The introduction of multivalent pneumococcal conjugate vaccines has led to changes on serotype distribution both in carriage and disease (Weinberger *et al.*, 2011). To understand the mechanisms leading to such changes, the study of pneumococcal multiple carriage is essential to distinguish between true serotype replacement and unmasking of nonvaccine types (Weinberger *et al.*, 2011). Serotyping of multiple colonies is the most straightforward approach to detect multiple carriage but is of little value due to the high cost and effort needed to achieve a satisfactory sensitivity (Huebner *et al.*, 2000). Several initiatives aimed to develop, refine and validate alternative methodologies to detect multiple carriage are ongoing (Satzke *et al.*, 2012).

We have recently studied multiple carriage among children in Portugal and how it has been affected by the 7-valent pneumococcal conjugate vaccine (Valente *et al.*, 2012b). We used a highly sensitive DNA capsular microarray that is able to detect and quantify over 90 serotypes. We observed that the distribution of the most abundant serotypes found in multiple carriage was similar to the one observed in single carriage or when only a single colony was studied.

Under the scope of another study of pneumococcal competence pherotypes (Valente *et al.*, 2012a), we have retrospectively identified 190 nasopharyngeal samples from which 2 pneumococcal serotypes had been detected based on colony morphology. These samples were extracted from a collection of 3406 samples obtained through cross-sectional studies carried out between 2001 and 2010 among young children. In those studies, 1 colony of each morphology was routinely picked from the agar plate.

We have now reviewed serotype distribution of the isolates present in the samples of the 190 co-colonized children and found that close to one-third of the isolates were either of serotype 3 (13.4%) or non-typeable (18.2%). The third most abundant type, 19F, accounted for 6.1% of the isolates. By contrast, the serotype distribution of the

entire collection of 3406 samples revealed that, overall, serotype 3 and non-typeable strains accounted for significantly fewer isolates: 4.9% and 5.3%, respectively (both *P*-values <0.001, χ^2 test). Indeed, the 3 most abundant serotypes among the 3406 samples were 19A (9.0%), 19F (8.2%), and 6A (6.6%). Clearly, non-typeable and serotype 3 strains were overrepresented in the collection of co-carried strains selected on the basis of colony morphology.

Experienced laboratory researchers know that serotype 3 and non-typeable isolates are easily distinguished on an agar plate from other pneumococci: the former for yielding colonies highly mucous, the latter for displaying minute (rough) colonies. Our observations may not be surprising but they show and provide a quantitative measure on how biased the analysis of serotype distributions can be if multiple carriage is detected based on colony morphology.

Taken together, these observations stress that detection of multiple carriage based on colony morphology does not reflect its real epidemiology, not only because it underestimates the true prevalence of these events (Valente *et al.*, 2012b), but also because it is prone to detect types that display highly distinctive colony morphologies resulting in their significant overrepresentation.

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Chapter V

Pherotypes of co-colonizing pneumococci among Portuguese children

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Contributions:

C. Valente did all the experimental work with the exception of the antimicrobial susceptibility testing and pneumococcal isolation, which were performed under the scope of previous studies.

Summary

A recent study showed that pneumococcal pherotypes often co-exist in the nasopharynx and suggested that the impact of pherotype-mediated fratricide on competition is limited. We determined the impact of pherotype (or competence stimulating peptide, [CSP]) on pneumococcal nasopharyngeal co-colonization. Of 184 nasopharyngeal samples yielding two serotypes, 39.9% contained CSP1 only, 12.6% CSP2 only and 47.5% had one strain of each pherotype. The observed proportions of concordant and discordant pherotypes (52.5% and 47.5%, respectively) were compared with the ones estimated (53.8% and 46.2%, respectively) and there were no significant differences ($p=0.9$, X^2 test). Our results support the hypothesis that there is a limited role of pherotype in co-colonization.

Introduction

Streptococcus pneumoniae (the pneumococcus) remains a major cause of morbidity and mortality worldwide. Nasopharyngeal colonization, which is asymptomatic, is particularly frequent among young children (O'Brien *et al.*, 2009).

Pneumococci are naturally competent for transformation and horizontal gene transfer (HGT) by homologous recombination is considered the main mechanism of evolution of this species (Spratt *et al.*, 2001). The nasopharynx, where co-colonization may occur, is the privileged niche for such events (Valente *et al.*, 2012). Evidence for *in vivo* intra- and inter-species HGT involving colonizing strains has been described (Barnes *et al.*, 1995; Hiller *et al.*, 2010).

In pneumococci, competence is triggered by the competence stimulating peptide (CSP), a pheromone that is prone to allelic variation (Havarstein *et al.*, 1995; Whatmore *et al.*, 1999). Two dominant pherotypes - CSP1 and CSP2 – have been

identified (Pozzi *et al.*, 1996; Ramirez *et al.*, 1997; Whatmore *et al.*, 1999). Each pherotype is only recognized by its specific membrane-associated histidine kinase ComD receptor that, upon binding of CSP, initiates competence (Havarstein *et al.*, 1996).

In recent years, it has been shown that pneumococci can compete in co-cultivation through a mechanism called fratricide, in which competent cells can kill their non-competent sisters (Guiral *et al.*, 2005). During competence an immunity membrane protein, ComM, which protects competent cells from their own hydrolases, is produced (Havarstein *et al.*, 2006). Bacteria not responding to the external CSP will not express ComM and will be killed by their competent siblings (Johnsborg *et al.*, 2008). It has been proposed that fratricide could provide access to nutrients, stabilize the relationship with the host through immune response, or drive genetic evolution through increased gene exchange (Havarstein *et al.*, 2006).

The role of pherotypes in competition and genetic differentiation remains a matter of debate. Specifically, whether or not it restricts recombination in the species is not totally clear. Some authors have proposed that the CSP/ComD specificity should imply a limited inter-pherotype recombination, resulting in two genetic populations associated with the two dominant pherotypes (Carrolo *et al.*, 2009; Claverys & Havarstein, 2007). Others argued that the CSP polymorphisms are determinant in driving pneumococcal genetic differentiation by acting as a starting point for genetic diversity (Cornejo *et al.*, 2010).

A recent study by Vestrheim *et. al*. has looked at pairs of co-colonizing strains to evaluate the impact of pherotype on the co-existence of pneumococci in the nasopharynx (Vestrheim *et al.*, 2011). The authors showed that pneumococcal pherotypes often co-exist in the nasopharynx and suggested that the impact of competence induced fratricide on competition is limited.

Considering this seminal and very recent observation, we aimed to determine the pherotype of pneumococcal strains from Portuguese co-colonized samples.

Materials and methods

Bacterial samples. Co-colonizing pneumococcal isolates (n=368) were selected from nasopharyngeal samples obtained from children in Lisbon and Oeiras, Portugal, in cross-sectional studies conducted in different years, between 2001 and 2010: 2001-2003, 2006-2007, and 2009-2010, as described before (Mato *et al.*, 2005; Nunes *et al.*, 2012; Sá-Leão *et al.*, 2009; Simões *et al.*, 2011). Identification of pneumococci was done as previously described (Mato *et al.*, 2005). Of 5,809 nasopharyngeal samples yielding a pneumococcal positive culture, 184 (3.2%) contained two strains selected on the basis of distinct colony morphology and posterior confirmation of distinct capsular types. Serotyping was done by the Quellung reaction using commercially available pneumococcal antisera (Statens Serum Institute, Copenhagen, Denmark) and/or by PCR as described previously (Pai *et al.*, 2006).

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed using the Kirby-Bauer technique, according to the Clinical and Laboratory Standards Institute (CLSI) recommendations and definitions (CLSI, 2008) for chloramphenicol, erythromycin, clindamycin, tetracycline, and sulfamethoxazole-trimethoprim (SXT). MIC of penicillin was determined with E-test (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations and interpreted according to CLSI guidelines for oral penicillin. Multidrug resistance was defined as resistance to three or more classes of antibiotics.

Pherotype assignment. Pherotype assignment was done by detection of CSP1 or CSP2 specific gene fragments, using a multiplex PCR described by Carrolo *et. al*.

which generates amplification products of 620 bp and 340 bp, respectively (Carrolo *et al.*, 2009).

Data analysis. Samples in which both pneumococcal strains were of the same pherotype were regarded as concordant and samples in which the co-colonizing pneumococci belonged to different pherotypes were regarded as discordant.

Co-colonization of pneumococcal pherotypes was assumed to occur independently and the multiplicative rule for independent events [Prob (A and B) = Prob (A) x Prob (B)] was used to estimate the distribution of concordant or discordant pherotypes, based on the overall distribution of pherotypes in the co-colonized samples, as described by Vestrheim *et. al*. (Vestrheim *et al.*, 2011). Statistical analyses were performed using X_1^2 test or Fisher exact test as appropriate.

Results

Of the 368 pneumococcal isolates all but one isolate were of either CSP1 or CSP2 corroborating previous studies on the dominance of these pherotypes on the population (Carrolo *et al.*, 2009; Cornejo *et al.*, 2010; Ramirez *et al.*, 1997). The single exception was obtained for a serotype 38 strain that probably expresses a different pherotype. For this reason, one co-colonized sample was excluded from further analyses. Of the 366 isolates remaining under study, 32 serotypes were represented. Nineteen capsular types contained representatives of both pherotypes (Figure 1). Serotypes 5, 7F, 9V, 11A, 14, 15B/C, 16F, 17F, 21, and 35F contained only representatives of CSP1; serotypes 9L/9N and 24F contained only representatives of CSP2 (Figure 1). Of all isolates, 63.7% were of pherotype CSP1 (n=233) and 36.3% were of pherotype CSP2 (n=133) reflecting the pherotype distribution and the predominance of CSP1

described in the literature for clinical isolates (Carrolo *et al.*, 2009; Cornejo *et al.*, 2010; Ramirez *et al.*, 1997).

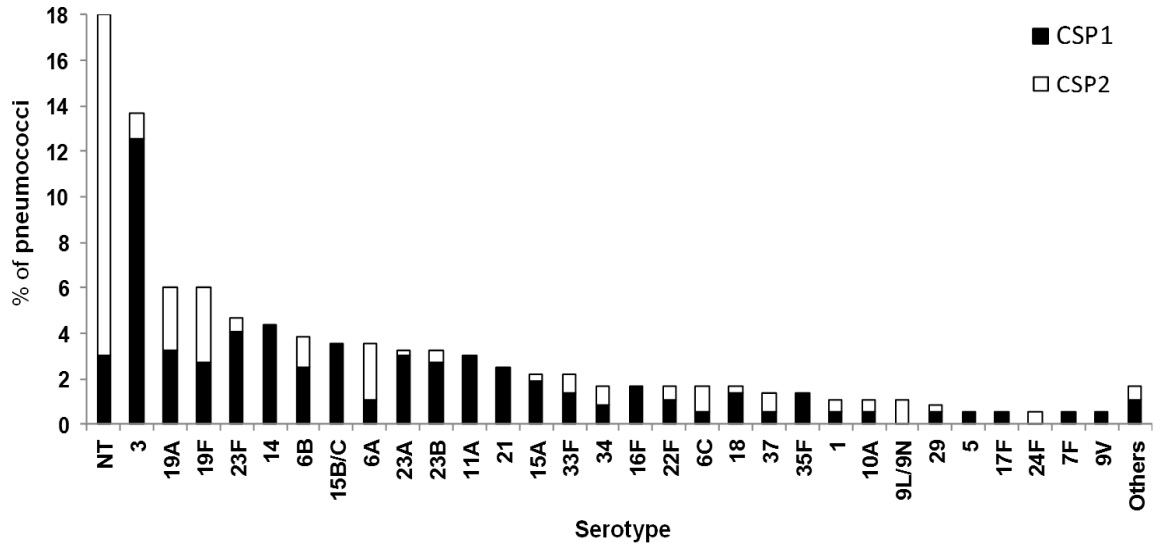

Figure 1. Pherotype and serotype distributions. Serotypes with an absolute frequency of 1 (i.e., serotypes 8, 9A, 31, 33A, 36, and 38) were grouped as Others. CSP, competence-stimulating peptide; NT, nontypeable.

Regarding antibiotic resistance, significant associations between pherotype CSP2 and resistance to at least one class of antibiotics, multidrug resistance, and individually with each of the antibiotics tested (with the exception of chloramphenicol) were observed (Table 1). As a substantial proportion (41%) of the CSP2 strains were non-typeable (NT) pneumococci, which are frequently drug-resistant (Sá-Leão *et al.*, 2006), the analysis was repeated excluding all NT pneumococci. Again, the strength of the association between CSP2 and resistance remained significantly higher compared to the strength of CSP1: multidrug resistance was 27.9% among CSP1 vs 42.3% among CSP2, p=0.02, X_1^2 test; and resistance to at least one antibiotic was 15.3% among CSP1 vs 38.5% among CSP2, p<0.0001, X_1^2 test). The exclusion of NT pneumococci also did not abolish the significant association of resistance to each antibiotic and pherotype CSP2.

Table 1. Association between antimicrobial resistance and pherotype.

Antimicrobial agent[a]	CSP1		CSP2		OR[b] (95% CI)	*p*
	Resistant	Susceptible	Resistant	Susceptible		
Penicillin G	48	185	68	65	0.25 (0.15;0.40)	<0.0001
Erythromycin	48	185	70	63	0.23 (0.14;0.38)	<0.0001
Clindamycin	44	189	62	71	0.27 (0.16;0.44)	<0.0001
Tetracycline	32	201	69	64	0.15 (0.09;0.25)	<0.0001
Chloramphenicol	3	230	3	130	0.57 (0.09;3.56)	0.38
SXT	28	205	57	76	0.18 (0.10;0.32)	<0.0001
MDR[c]	38	195[d]	70	63[d]	0.18 (0.11;0.31)	<0.0001
Resistant ≥1[e]	80	153	83	50	0.32 (0.20;0.50)	<0.0001

SXT- sulfamethoxazole-trimethoprim; MDR – multidrug resistant

[a]Intermediate and resistant isolates were considered resistant for the analysis.

[b]An OR of > 1, indicates a significant association of CSP1 with resistance to the antimicrobial agent, whereas an OR of < 1 indicates a significant association of CSP2 with resistance to the antimicrobial agent.

[c] Multidrug resistance, resistance to three or more classes. The most common profile was resistance to penicillin, erythromycin, clindamycin, tetracycline and SXT (2.6% of CSP1 strains and 30% of CSP2 strains).

[d]Includes all non-MDR strains

[e]Resistant to at least one class.

Among the co-colonized samples, 39.9% contained two strains of CSP1, 12.6% contained two strains of CSP2, and 47.5% had one sample of each pherotype. When the observed and estimated proportions of concordant and discordant pherotypes in the co-colonized samples were compared no significant differences were observed

(p=0.9, X^2 test) (Table 2), suggesting that co-colonization of pherotypes CSP1 and CSP2 occurs independently.

Table 2. Distribution of pherotypes among co-colonized samples.

Pherotypes	Estimated proportion (%)	Observed proportion	
		No. samples	Proportion (%)
CSP1 and CSP1	40.6	73	39.9
CSP2 and CSP2	13.2	23	12.6
CSP1 and CSP2	46.2	87	47.5
Concordant	53.8	96	52.5
Discordant	46.2	87	47.5

As the serotype distribution in our samples was characterized by a predominance of NT pneumococci (18.0%) and strains of serotype 3 (13.7%) (Figure 1), which could potentially bias the results, we repeated the analysis after exclusion of these strains. The results indicated that, again, the observed and estimated proportions of concordant (55.6% and 56.6%, respectively) and discordant (44.4% and 43.4%, respectively) pherotypes were not significantly different (p=0.8, X^2 test). This confirmed the results obtained with all samples regarding the independent co-existence of pneumococcal pherotypes in the nasopharynx.

Discussion

The results obtained in our study replicate the observations by Versthreim *et. al.* regarding the independent co-existence of different pherotypes in co-colonization.

In addition, we report a significant association of pherotype CSP2 with antimicrobial resistance. As no information on the genetic backgrounds of the strains was obtained, the possibility that these findings might result from high clonality between the resistant isolates could be raised. In fact, Carrolo *et al*. observed that pherotype is a clonal property and is not randomly dispersed within the pneumococcal population (Carrolo *et al.*, 2009). Nevertheless, given the high genetic diversity of the pneumococcal population, the high assortment of serotypes in our collection, and the fact that the samples were collected in a wide temporal range (2001-2010), clonal diversity within each pherotype is expected.

Of interest, the general association between CSP2 and antibiotic resistance among Portuguese isolates from carriage contrasts with the observation that among Portuguese isolates from invasive disease an association was described between penicillin resistance and CSP1(Carrolo *et al.*, 2009). However, this latter collection was obtained in a shorter time period (1999-2002) and before widespread use of conjugate vaccines occurred in the country (Aguiar *et al.*, 2008; Sá-Leão *et al.*, 2009; Simões *et al.*, 2011) that have resulted in major changes among circulating pneumococci. In addition, an asymmetric distribution of circulating lineages among carriage and disease is also expected (Sá-Leão *et al.*, 2011) and may also have accounted for these contrasting observations.

Our study (as the one from Verstrheim *et al.* (Vestrheim *et al.*, 2011)) has some limitations. Firstly, samples originated from cross-sectional studies and, therefore, duration of carriage was not taken into account (Gray *et al.*, 1980). Whether detection of co-colonization results, in general, from a true co-existence event or reflects mostly a transitional state remains to be ascertained. Secondly, the relative abundance of the co-colonizing strains was not quantified. One can imagine that if the proportion of two strains of different pherotypes in the host is very different, competition may be

occurring despite their co-existence. Thirdly, we used a convenience sample based on the detection of samples where pneumococcal colonies exhibited different morphologies. Hence, it is anticipated that we underestimated co-colonization events in our collections and that only a subset of it was analyzed. Fourthly, we did not attempt to measure whether the isolates had the capacity to become competent and induce fratricide. Such approach, could, however, be still inconclusive, as lack of capacity to induce competence in vitro does not necessarily imply the strain is unable to undergo competence in vivo (Pozzi *et al.*, 1996; Ramirez *et al.*, 1997; Whatmore *et al.*, 1999).

Still, it is of particular interest that in both studies – ours and the one from Verstrheim *et al.* (Vestrheim *et al.*, 2011) - using collections from different years and geographical origins, the same conclusions were reached, suggesting a low impact of CSP-mediated competition on pneumococcal co-existence in the nasopharynx. Johnsborg *et al*. (Johnsborg *et al.*, 2008) have previously reported the existence of ComD promiscuity between *S. pneumoniae* and closely-related species, which might allow target bacteria to sense a non-cognate CSP resulting in the expression of ComM immunity protein. To our best knowledge this cross talk has not been found to occur between pneumococcal isolates producing CSP1 or CSP2 and their corresponding ComD1 and ComD2 proteins. This finding would, however, support the hypothesis that CSP-mediated competition has little impact on pneumococcal co-existence.

In summary, our data support the hypothesis that pherotype-mediated fratricide does not seem to be an important mechanism of within-host competition. However, further studies of longitudinal design with systematic detection of co-colonization and assessment of colonization density are needed to provide further insights on the nature of pneumococcal co-colonization.

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Author disclosure statement

The authors have no competing interests to disclose.

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Chapter VI

Characterization of the *blp* locus of co-colonizing pneumococci and its impact on co-colonization

In prepraration:

Valente, C., S. Dawid, F. R. Pinto, J. Hinds, A. S. Simões, K. A. Gould, L. A. Mendes, H. de Lencastre, and R. Sá-Leão. Characterization of the *blp* locus of co-colonizing pneumococci and its impact on co-colonization.

Contributions:

C. Valente was responsible for all experimental work with the exception of the comparative genomic hybridization experiments, which were performed by K. Gould and analyzed by J. Hinds, and 90% of MLST genotyping, which was performed by A. S. Simões. The process of strain isolation resulted from team-work between C. Valente, A. S. Simões and L. A. Mendes.

Summary

Abstract: Bacteriocins are small antimicrobial peptides secreted by bacteria. In *Streptococcus pneumoniae*, a frequent pathobiont inhabiting the human nasopharynx, the *blp* (bacteriocin-like peptides) locus is involved in the regulation and secretion of bacteriocins and has been implicated in inter-strain competition *in vivo*. In this study we aimed to evaluate the impact of the *blp* locus and phenotypes of bacteriocin secretion carriage of multiple pneumococcal strains.

We used a collection of 135 nasopharyngeal samples from individuals carrying two or more pneumococcal strains characterized through serotyping, MLST genotyping, and assignment of the type of competence stimulating peptide (CSP). The *blp* locus of all isolates was characterized genetically with regards to pheromone type, locus functionality and bacteriocin immunity content. Phenotypes of bacteriocin secretion and locus activity were assessed through overlay competition assays. Isolates from single carriage events (n=298) were also characterized for comparison.

Genetic analysis of the *blp* locus revealed that co-colonizing pneumococci present a high diversity of *blp* cassettes, although conservation is essentially maintained among related strains. Close to one third of co-colonizing strains showed inhibitory activity against a control susceptible strain and over half were inactive. Pneumococci co-colonized individuals independently of their *blp* pherotype (p=0.577), locus activity (p=0.798) and inhibitory phenotype (p=0.716). Characterization of isolates from single carriage revealed no differences when compared to co-colonizing pneumococci.

Despite clear evidence of *blp* mediated competition in experimental models, our large scale study of natural co-colonizing strains suggests that the *blp* locus plays a limited role in selecting which strains can colonize the nasopharynx simultaneously.

Importance: Nasopharyngeal colonization with *Streptococcus pneumoniae* (pneumococcus) is important for pneumococcal evolution as it represents an

opportunity for horizontal gene transfer when multiple strains co-occur, a phenomenon known as co-colonization. Understanding how pneumococcal strains interact within the competitive environment of the nasopharynx is of chief importance in the context of pneumococcal ecology. In this study we showed that a biological process frequently used by bacteria for competition is not determinant in the co-existence of pneumococcal strains in the host, contrary to what has been shown in experimental models. To our knowledge this is the first time that an unbiased collection has been used to measure the impact of *blp*-bacteriocin production on competition in the nasopharynx.

Introduction

Streptococcus pneumoniae is an important bacterial pathogen causing infectious diseases with a high rate of mortality worldwide, particularly among young children, the elderly and the immunocompromised (CDC, 2012; CDC, 2014; O'Brien *et al.*, 2009). Despite the significant morbidity and mortality attributed to this pathogen, disease is rare compared with the rate of asymptomatic nasopharyngeal colonization (Bogaert *et al.*, 2004).

Nasopharyngeal colonization with *Streptococcus pneumoniae* is prevalent, in particular among young children. Co-occurrence of different pneumococcal strains in the human nasopharynx, also known as co-colonization or multiple serotype carriage, is also frequent, reaching up to 50% of all individuals (Brugger *et al.*, 2010; Turner *et al.*, 2011; Valente *et al.*, 2012b; Wyllie *et al.*, 2014). Co-colonization represents an opportunity for horizontal gene transfer and is, therefore, an important event for pneumococcal evolution (Barnes *et al.*, 1995; Hiller *et al.*, 2010).

Little is known about how different pneumococcal strains interact within the nasopharynx, but data from longitudinal studies has shown that the pneumococcal population colonizing children is in constant turnover. Also, the serotype replacement that was observed after the introduction of anti-pneumococcal vaccination suggests that a biological mechanism of competition between serotypes was occurring prior to vaccination, maintaining the replacing serotypes at a low level in the population (Auranen *et al.*, 2009; Lipsitch *et al.*, 2000). Moreover, the fact that the pneumococcus natural niche is a dense polymicrobial environment, with limited resources, highlights the importance of competitive interactions in its lifestyle (Garcia-Rodriguez & Fresnadillo Martinez, 2002)

A number of molecular mechanisms have been shown to be involved in pneumococcal intra-species competition, namely, competence-mediated fratricide and bacteriocin production, *i. e*., production of antimicrobial peptides (Dawid *et al.*, 2007; Guiral *et al.*, 2005; Havarstein *et al.*, 2006; Reichmann & Hakenbeck, 2000).

Fratricide occurs during the competent state, in which there is expression of a hydrolase, CbpD, and an immunity protein, ComM, which protects competent cells against their own lysis. It is through the induction of CbpD and a number of autolysins that immune competent cells kill non-competent (therefore, susceptible) cells sharing the same niche. In this way, competent cells not only eliminate direct competitors, but also benefit from compounds released by non-competent lysed cells (Havarstein *et al.*, 2006).

We and others have previously addressed the impact of the competence stimulating peptide (CSP) pherotype on the co-existence of pneumococci, looking at pairs of co-colonizing strains (Valente *et al.*, 2012a; Vestrheim *et al.*, 2011). Both studies have shown that pneumococcal strains can co-exist in colonization independently of their

CSP type, suggesting a limited impact of CSP-mediated competition on co-colonization.

In *S. pneumoniae,* bacteriocin production associated with the *blp* locus has been shown to play a role in intraspecies competition in a murine model of colonization (Dawid *et al.*, 2007). The *blp* locus encodes a number of bacteriocins whose secretion is controlled by a typical two component regulatory system, constituted by an histidine kinase (BlpH) and a response regulator (BlpR), and activated upon binding of the signalling peptide BlpC to its cognate BlpH receptor (Dawid *et al.*, 2007; Reichmann & Hakenbeck, 2000). Activation of the response regulator BlpR results in the upregulation of the entire locus (de Saizieu *et al.*, 2000), including the bacteriocin/immunity region (BIR) that encodes the bacteriocins (at least sixteen, several with allelic variability) and their cognate co-transcribed immunity proteins (Bogaardt *et al.*, 2015; Son *et al.*, 2011). Bacteriocins (and BlpC) are secreted by the ABC transporter BlpAB (Dawid *et al.*, 2007; de Saizieu *et al.*, 2000; Reichmann & Hakenbeck, 2000).

Besides the genetic variability of the BIR, at least 5 types of the peptide pheromone BlpC have been found, with minor variants among each type (Bogaardt *et al.*, 2015; de Saizieu *et al.*, 2000; Reichmann & Hakenbeck, 2000; Son *et al.*, 2011). In addition, a disruption in the *blpA* gene has been shown to render strains with an otherwise intact *blp* locus unable to secrete both the peptide pheromone and the bacteriocin peptides. These strains display a *cheater* phenotype as they are able to recognize exogenous BlpC, resulting in the expression of immunity proteins without the cost of peptide secretion (Son *et al.*, 2011). The universal conservation of the *blp* locus in pneumococcal genomes and the significant diversity of content suggest that the locus provides a competitive advantage during either colonization or transmission. No studies to date have examined whether *blp* locus activity restricts co-colonization to only non-antagonistic interactions.

In this study we identified and characterized genotypically and phenotypically a collection of co-colonizing pneumococci in order to explore the role of the *blp* locus and bacteriocin secretion on the co-existence of *S. pneumoniae* in colonization. The results with this strain collection were compared with those from a collection of single carriage strains to address possible differences between strains from single and co-colonization events.

Materials and methods

Sample selection and inclusion criteria. Nasopharyngeal samples collected from healthy children attending day-care centers in Oeiras and Montemor-o-Novo, Portugal, were retrospectively selected from cross-sectional studies conducted between 2001 and 2010: 2001, 2006-2007 and 2009-2010 (Mato *et al.*, 2005; Nunes *et al.*, 2012; Sá-Leão *et al.*, 2009; Simões *et al.*, 2011a). Samples were selected with the objective of detecting the highest number possible of co-colonization events. Selection was based on the previous observation that the serotypes found more frequently in single and multiple carriage events are essentially the same (Valente *et al.*, 2012b). In the selected years, the serotypes with highest cumulative prevalence were: 3, 6A, 6C, 11A, 15A, 15B/C, 19A, 19F, 21, 23A, 23B and non-encapsulated pneumococci (NT). All samples yielding a pneumococcal positive culture for one of these serotypes in which a primary selective plate frozen stock was available (see below) were selected for molecular detection of co-colonization, as described below (n=1,415).

Sample collection, isolation of pneumococci and serotyping. Nasopharyngeal samples were collected by pediatric nurses using sterile mini-tip calcium alginate swabs and plated on the same day in a primary selective plate of 5% blood trypticase soy agar containing 5mg/L gentamicin to select for *S. pneumoniae*. Plates were incubated overnight at 37ºC under anaerobic conditions with an optochin disk. Swabs

were frozen at -80ºC in 1mL Mueller-Hinton broth with 30% glycerol, with the exception of the swabs from 2001-2003 that were discarded. On the second day, pneumococcal colonies with different morphologies were picked and plated separately (one colony per morphology). The bacterial lawn of the primary selective plate was collected and frozen, as described above, in samples collected during or after 2006 and in a subset of samples collected during 2001. On the third day, pneumococcal isolates were frozen.

Pneumococcal strains were serotyped by multiplex PCR, as described previously (Brito *et al.*, 2003; Pai *et al.*, 2006), http://www.cdc.gov/streplab/pcr.htm]. Strains that could not be typed by PCR were serotyped by the Quellung reaction (Sorensen, 1993) using commercially available pneumococcal antisera (Statens Serum Institute, Copenhagen, Denmark).

DNA isolation. Total DNA was isolated from 200µL of the primary selective culture frozen stock using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. DNA was eluted in 100µL milli-Q water (Millipore Corporation, Billerica, MA, USA).

Detection of co-colonization and capsular typing of the co-colonizing strains. Detection of co-colonization was done as previously described (Brugger *et al.*, 2009). Briefly, the noncoding region between the pneumolysin gene and the preceding hypothetical protein gene was amplified and digested with up to four restriction enzymes (AflIII, ApoI, DdeI MseI). Fragments were separated by capillary electrophoresis and their sizes were determined. Co-colonization was assumed whenever the sum of the size of the digestion fragments was higher than the size of the undigested PCR product.

The putative co-colonized samples were analyzed using the BuG@S SP-CPSv1.4.0 microarray for molecular serotyping of the co-colonizing strains, as described

previously (Brugger *et al.*, 2010). Statistical analysis of the microarray data to determine the serotypes present in a sample and their relative abundances was done as described in (Newton *et al.*, 2011).

All serotypes identified by the microarray were confirmed by PCR using as template purified DNA of the primary selective growth.

For the interpretation of the microarray results, a serotype was considered a major serotype whenever its relative abundance was ≥70%. Accordingly, a serotype was considered a minor serotype whenever its relative abundance was ≤30%. Serotypes with relative abundances >30% and <70% were considered co-dominant.

Isolation of pneumococcal strains from the co-colonized samples. To isolate co-colonizing pneumococcal strains present in the nasopharyngeal samples 50µL aliquots of the primary selective plate frozen stock were prepared, serially diluted and plated in 5% blood trypticase soy agar containing 5mg/L gentamicin plates to obtain isolated colonies. On the following day, individual colonies were picked for amplification of serotype-specific capsular genes, using the set of primers described above (Brito *et al.*, 2003; Pai *et al.*, 2006), [http://www.cdc.gov/streplab/pcr.htm]. The number of colonies picked per strain was calculated using the binomial formula described by Huebner *et al*. (Huebner *et al.*, 2000) $q^n=(1-P)$, where q=1-[concentration of the serotype], P=probability of finding one or more colonies of that serotype and n=number of colonies needed to detect the serotype with the assumed probability. For the calculations, a probability of 95% was considered and the relative abundances determined by the CPS microarray for each serotype in each sample were used. One colony positive for each capsular type was subcultured and frozen at -80ºC in 1mL Mueller-Hinton broth with 30% glycerol. For isolation and correct identification of non-encapsulated pneumococci the method described by Simões *et al.* was used (Simões *et al.*, 2011b).

MLST. All co-colonizing strains were genotyped by multi locus sequence typing (MLST), performed by amplification of internal fragments of seven housekeeping genes (*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl*), as described previously (Enright & Spratt, 1998). Sequencing reactions were conducted at Macrogen, Inc. (Amsterdam, Netherlands) and at STAB VIDA. Lda (Caparica, Portugal). Sequence analysis was performed either with the Bionumeris software (Applied Maths, Gent, Belgium) or with the DNAStar software (DNASTAR, Inc. Madison, WI, USA). Assignment of allele numbers and sequence types (ST) was done at the MLST database for *S. pneumoniae* (http://pubmlst.org/spneumoniae/).

Interpretation of results was performed with the PHYLOViZ platform that uses the goeBURST algorithm to determine possible evolutionary relationships between isolates (Francisco *et al.*, 2012). Level two was chosen in the goeBURST configuration for computing the groups, *i.e*., strains sharing 5 out of 7 alleles were considered genetically related. Clonal complexes (CC) were assigned considering only the collection of co-colonizing pneumococci.

Assignment of pherotype CSP1 or CSP2. Pherotype assignment was done by multiplex PCR amplification of specific *comC* gene fragments, as described previously (Carrolo *et al.*, 2009).

PCR assignment of *blpC* type. Assignment of the five *blpC* types described up to now was done by amplification of specific *blpC* gene fragments, using primers designed for this study (Table S1). The PCR reactions were done in a 10µl volume with 1x GoTaq Flexi Buffer (Promega, Madison, USA), 0.12 mM concentration of dNTPs, 1.0µM concentration of each primer, and $1U\mu l^{-1}$ of GoTaq Flexi DNA Polymerase (Promega, Madison, USA). $MgCl_2$ concentration varied according to primer pair (Table S1). DNA was obtained from freshly grown bacterial cultures picked with a sterile tip and briefly immersed in the PCR reaction mix. Thermocycling was performed with the following

conditions: 95ºC for 5 min; 30 amplification cycles of 95ºC for 30s, 52-58ºC (Table S1) for 30s and 72ºC for 45s; and a final extension step at 72ºC for 5 min. PCR analysis was done by electrophoresis on 1% Seakem LE agarose gels stained with Ethidium Bromide in 1x TAE buffer.

Assessment of *blpA* integrity. Detection of the 4 bp repeat insertion was done by PCR amplification of a *blpA* gene fragment containing the region of repeat insertion using forward primer 21 described in (Son *et al.*, 2011) and reverse primer with sequence AGCCGCTGATGAAATGGGC, followed by digestion with Cac8I (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions. Restriction occurs only when the 4 bp repeat is present.

Samples in which we failed to amplify the region containing the repeat insertion were amplified with primers 18 and 19 described in (Son *et al.*, 2011) for amplification of the total *blpA* gene to confirm presence of possible deletions, by comparison of the size of the PCR product with that of a control strain with an intact *blpA* gene.

RFLP analysis of the bacteriocin/immunity region (BIR) and bacteriocin content prediction. RFLP profiles of all isolates were determined as previously described (Son *et al.*, 2011). Briefly, PCR products were obtained with primers 1 and 2, purified and digested with AseI (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions. Digestion products were analyzed by capillary electrophoresis and isolates with identical restriction patterns were assigned to the same group.

BIR content was predicted by comparison of RFLP profiles with those of sequenced strains available at GenBank, using either VectorNTI® or CLC Genomics softwares. Overlay assays with reporter strains of known BIR contents (Table 1) were performed as a control for *in silico* predictions.

Table 1. Reporter strains for characterization of the *blp* locus

Strain designation	Strain characteristics	Reference
P537	Serotype 6A strain with deletion of *blpRHCBA-BIR*. Susceptible to all bacteriocins secretion. Reporter of inhibitory activity	(Son *et al.*, 2011)
PSD121	R6 background; insertion of a type P164 *blp* locus, with *blpC* deletion and *lac-Z* reporter under control of the BIR promoter. Type P164 BlpC/BlpH reporter	(Kochan & Dawid, 2013)
PSD101	R6 background; insertion of a type R6 *blp* locus, with *blpC* deletion and *lac-Z* reporter under control of the BIR promoter. Type R6 BlpC/BlpH reporter	(Pinchas *et al.*, 2015)
PMP105	R6 background; insertion of a type 6A *blp* locus, with *blpC* deletion and *lac-Z* reporter under control of the BIR promoter. Type 6A BlpC/BlpH reporter	(Pinchas *et al.*, 2015)
PMP105	R6 background; insertion of a type T4 *blp* locus, with *blpC* deletion and *lac-Z* reporter under control of the BIR promoter. Type T4 BlpC/BlpH reporter	(Pinchas *et al.*, 2015)
P1	Serotype 6A, BIR with MNO, $BlpC_{6A}$. Control for MNO BIR content	(Kim & Weiser, 1998)
P4	Serotype 6B, BIR with QMNO, $BlpC_{P164}$. Control for QMNO BIR content	(Kim & Weiser, 1998)
P132	Serotype 29, BIR with K, type 3 $BlpC_{6A}$. Control for K BIR content	(Son *et al.*, 2011)
P133	Serotype 6A, BIR with IJK MNO, $BlpC_{R6}$. Control for IJK MNO BIR content	(Son *et al.*, 2011)
P140	Serotype 35B, BIR with IJK, $BlpC_{R6}$. Control for IJK BIR content	(Son *et al.*, 2011)
P155	Reference for sequencing analysis of the *blpC* gene of strains with $blpC_{P155}$ type.	(Son *et al.*, 2011)

Overlay assays. Inhibition and activity overlay assays were performed as described elsewhere (Son *et al.*, 2011) and using the reporter strains listed in Table 1. Briefly, strains were grown overnight on 5% blood trypticase soy agar (TSA) plates, colonies were collected with a tip and inserted into TSA plates supplemented with ≈ 4,700U catalase, followed by a 6h incubation at 37ºC in 5% CO_2. Overlay reporter strains were grown at 37ºC in Todd Hewitt broth supplemented with yeast extract (THY) to an OD_{620}

of 0.5. Two hundred microliters of culture were added to a 15 ml conical tube containing 5ml of THY, 3mL of molten TSA, ≈ 4,700U catalase, and 50µl of X-Gal at 40mg/ml (for signaling overlays only). The mixture was applied over the TSA plates containing the pre-grown isolates, followed by an overnight incubation at 37ºC in 5% CO_2. Plates were inspected for halos of growth inhibition (inhibition overlays) or β-galactosidase activity (signaling overlays). Each overlay assay was performed twice on two different days, with at least two technical replicates in every assay.

Data analysis. As our collection of co-colonized samples included a number of samples with three pneumococcal strains, for the data analysis dual interactions of pneumococcal strains were considered: samples with two strains accounted for one interaction and samples with three strains accounted for three interactions.

For each comparison, interactions of strains with the same genotypic and/or phenotypic characteristic were regarded as concordant and interactions of strains with different genotypic and/or phenotypic characteristic were regarded as discordant.

The frequency of inhibitory activity, serotypes, CSP, BlpC and BlpA types were compared between strains found in co-colonization and strains found in single colonization samples. Frequency data was organized in contingency tables where rows were defined by different types and columns by single and co-colonization sets of strains. To detect statistically significant differences, 5000 random contingency tables were generated by randomly permuting the strain's row and column label. This process generates tables with constant row and column totals that are consistent with the null hypothesis that row distribution is independent of column membership. A quantile q for each individual frequency in the contingency table was computed as the fraction of random tables with a lower frequency. If $q>0.5$, then the corresponding two-tail p-value is $2(1-q)$, otherwise the p-value is $2q$. The deviation of the complete contingency table from the null hypothesis was also evaluated through a Fisher's Exact Test. A similar

analysis was performed to compare the sets of strains found in co-colonizing samples classified as minor, co-dominant or major, according to their relative abundance.

The frequency with which pairs of serotypes (or of CSP, BlpC and BlpA types) appear together in co-colonizing samples was also compared with a null hypothesis where strains mix randomly. The frequency distribution under the null hypothesis was estimated through 5000 random assignments of strains to the samples with co-colonization events. This random assignment maintained the number of different strains detected in each sample. Additionally, each sample had to contain at least one strain from the set of serotypes used initially to select the samples included in this study (3, 6A, 6C, 11A/D, 15A, 15B/C, 19A, 19F, 21, 23A, 23B, NT). A quantile q for each possible pair type frequency was computed as the fraction of randomizations with a lower frequency. If $q>0.5$, then the corresponding two-tail p-value is $2(1-q)$, otherwise the p-value is $2q$. A similar analysis was performed to compare the pair type frequency among samples with major/minor versus co-dominant co-colonization events.

Frequency deviations were considered significant when p-values<0.05.

Results

Detection of co-colonization and isolation of pneumococcal strains from co-colonized samples. Out of the 1,415 samples included in the study, 362 samples were excluded after the *ply*NCR amplification, due to insufficient pneumococcal DNA in the sample. The combination of the plyNCR-RFLP with the capsular microarray indicated the presence of two or more pneumococcal strains in 162 out of 1,053 samples (15.4%). Serotypes detected by the microarray were confirmed by PCR.

The capsular microarray identified 358 putative pneumococcal strains in the 162 co-colonized samples. By serial dilutions of the total bacterial growth, colony PCR serotyping, and confirmation of the colonies obtained as *bona fide* pneumococci we obtained 135 co-colonized samples with more than one pneumococcal strain isolated (further details in Figure S1). Of these, 120 samples contained two pneumococcal strains (88.9%) and 15 samples contained three pneumococcal strains (11.1%). This collection was used in the following analyses.

***S. pneumoniae* strains co-colonizing individuals are highly diverse and co-exist independently of the capsular type and genotype.** The total collection of co-colonized samples (n=135) contained 285 pneumococcal strains and was highly diverse. Thirty-six capsular types were found and MLST genotyping clustered strains in 19 clonal complexes (CC, CC1-CC19) and 36 singletons (S, S1-S36) (Figure 1).

A permutation analysis to test if serotypes or genotypes were found in co-colonized carriers at frequencies different than the ones expected by chance showed that serotypes 3 and NT were found in co-colonization at a lower frequency than expected ($p<0.0001$ for both serotypes) while serotype 15B/C was found in co-colonization events at a higher frequency than expected ($p=0.0342$). No statistical significance was reached when the analysis was repeated for genotypes.

Due to the high number of serotypes present in the collection, the frequency in which specific pairs of serotypes co-occurred was low, although we were able to find positive associations between pairs of serotypes (*i.e.*, pairs that co-occurred at a significantly higher frequency than the one expected based on the distribution of those serotypes in the population: NT-21 ($p=0.027$), 6C-19A ($p=0.046$), 16F-21 ($p=0.003$), 23F-19F ($p=0.017$), and 22F-3 ($p=0.036$).

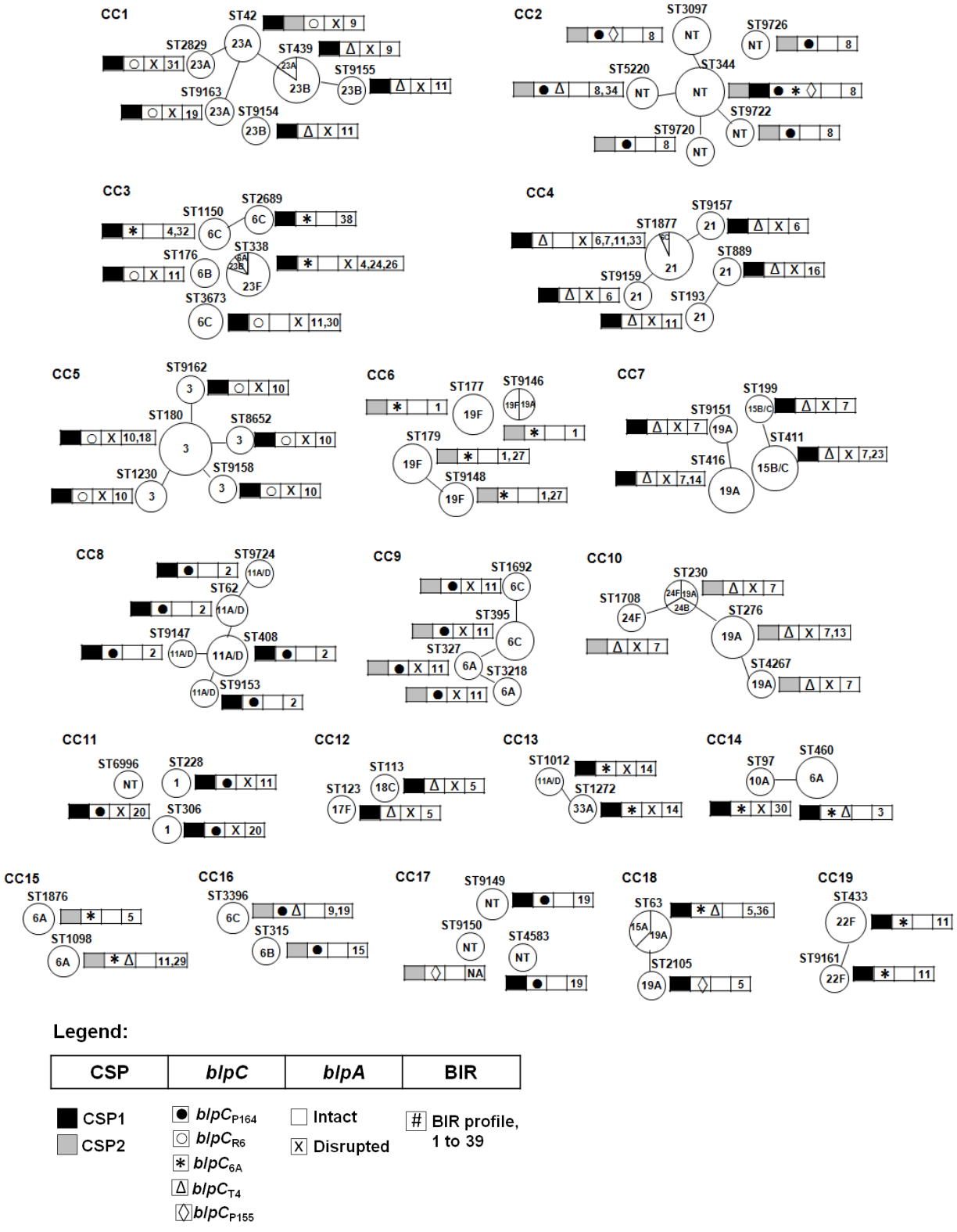

Figure 1. Genetic diversity and characteristics of the blp locus of co-colonizing pneumococci. Only clonal complexes are represented. RFLP profiles mached to genome sequenced strains (GenBank designations): 1 (GA13723), 2 (AP200), 3 (GA13856), 4 (GA54354), 5 (20703335), 7 (GA47439), 9 (670-6B), 10 (OXO141), 11 (GA17971), 14 (46518), 18 (SP14-BS69), and 19 (GA47502). *RFLP profiles with no match in GenBank.

Accordingly, several positive associations between specific genotypes were also found: CC7-CC1 (p=0.03), CC7-S19 (p=0.006), CC7-S14 (p=0.04), CC4-S12 (p=0.002), CC4-S27 (p=0.003), CC5-CC19 (p=0.03), and S12-S27 (p=0.01). Of note, MLST analysis revealed 24 novel STs, 10 of which were originated by the presence of new alleles. The remaining 14 were the result of novel allele combinations. Among the latter, for three STs (ST9160, ST9162 and ST9164) the allele separating the novel ST from its SLV was shared with its co-colonizing strain, suggesting that within host recombination might have occurred with co-colonizing strain (Table 2).

Table 2. Evidence for putative horizontal gene transfer in vivo in NP samples characterized in this study.

NP sample	ST	aroE	gdh	gki	recP	spi	xpt	ddl	CSP
Sample 8043									
8043-19A	**9160**	**1**	60	9	8	6	3	29	CSP1
8043-6C	395	**1**	5	7	12	17	1	14	CSP2
8043-31	1766	**1**	5	29	1	46	14	18	CSP2
Closest ST[a]	1151	7	60	9	8	6	3	29	-
Sample 8058									
8058-3	**9162**	**2**	15	2	10	6	1	22	CSP1
8058-23A	**9163**	**2**	8	9	9	6	4	6	CSP1
Closest ST[a] (3)	180	7	15	2	10	6	1	22	-
Closest ST[a] (23A)	190	8	8	9	9	6	4	6	-
Sample 8169									
8169-NT	**9164**	**7**	8	4	18	15	4	31	CSP2
8169-6C	1150	**7**	25	8	6	25	6	8	CSP1
Closest ST[a]	5516	2	8	4	18	15	4	31	-

[a] Closest ST found in the MLST database (http://spneumoniae.mlst.net/). NP, nasopharyngeal; ST, sequence type; CSP, competence stimulating peptide.

CSP assignment of all strains showed that 35.2% of the interactions occurring in the 135 co-colonized samples were between two strains of CSP1, 10.9% were between two strains of CSP2, and 51.5% were between strains of different pherotypes (CSP1, CSP2, and other type present in strains from serotype 38). When the frequency in which CSP types appear together in co-colonization was compared with the frequency

estimated under the hypothesis that strains mix randomly, the results suggest a tendency for co-colonization with strains of different CSP types, although with low robustness (p=0.048).

Despite the positive associations found at the serotype and genotype levels, the large diversity of our collection seems to suggest that pneumococcal strains co-colonize the human nasopharynx independently of their capsule, genetic background and CSP type.

Genetic characterization of the *blp* locus of co-colonizing pneumococci reveals high diversity of *blp* cassettes. The genetic characterization of the *blp* locus of the co-colonizing strains was focused on the assignment of the *blpC* type and in the assessment of the *blpA* integrity (presented later in the Results section), and in the RFLP analysis of the BIR. The results are summarized in Figure 1.

Thirty-six RFLP profiles were identified in the co-colonized collection, from which 16 could be assigned to known BIR sequences available in GenBank and accounted for 71.2% (n=203) of the strains. The remaining 23 profiles corresponded to not yet described BIR regions and accounted for 25.3% (n=72) of the strains. In ten strains (3.5%) we were not able to amplify the BIR region, despite several attempts. Of note, some BIR profiles, such as profiles 7 and 11, were detected in many clonal complexes (CC). Other profiles, highly prevalent as well, were restricted to a single CC, such as profiles 10 and 8, associated with CC5 and CC2, respectively. Our BIR predictions indicated that the number of bacteriocin peptides present in the co-colonizing strains varied between 2 and 6. Interestingly, profiles predicted to contain the highest number of bacteriocin peptides (profiles 7 and 11) were associated mostly with strains with a disrupted *blpA*, *i.e.*, *cheater* phenotype.

In agreement with previous studies, within each CC, the BIR region displayed a higher level of diversity, when compared to *blpA* and *blpC.* Nevertheless, taking into account

the 3 regions of the locus, our results show that there is conservation of the locus among related strains.

Pheromone peptides BlpC are not equally distributed in the population and do not restrict co-colonization. Co-existence of strains secreting different BlpC peptides implies that the strain at higher cell density would activate their *blp* locus earlier and be at a competitive advantage. To assess if the type of BlpC could prevent or facilitate co-existence of pneumococci in the host we determined the *blpC* allelle of the 285 co-colonizing strains. The results showed that 36.8% of the strains were of type $blpC_{T4}$, 22.5% of type $blpC_{P164}$, 22.1% of type $blpC_{6A}$, 16.5% of type $blpC_{R6}$, and 2.1% of type $blpC_{P155}$. These results suggest that *blpC* types are not equally distributed in the pneumococcal population, with $blpC_{T4}$ being significantly more prevalent and $blpC_{P155}$ significantly less prevalent (Figure 2).

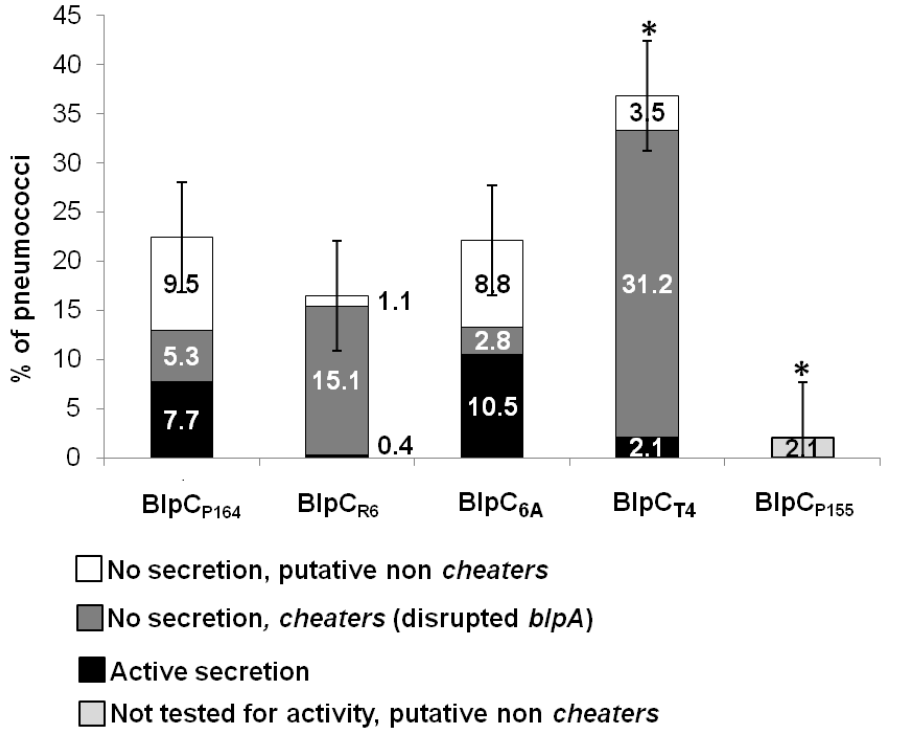

Figure 2. Distribution of *blpC* types in the co-colonization strains. Asterisks indicate statistically significant differences. Error bars represent standard error.

To confirm the result obtained by PCR, signaling overlay assays were performed for all strains using reporter strains for each BlpC type (Table 1). The BlpC type was confirmed in all strains in which it was possible to confirm locus activity, as indicated by the β-galactosidase activity in the assays (n=59). However, there was a significant proportion of strains in which we were not able to confirm phenotypically the secretion of the specific BlpC type (79%).

Since no reporter strain was available for *blpC*$_{P155}$, all strains in which this *blpC* type was detected by PCR were confirmed to be of type P155 by sequencing of the PCR product and comparison with that of the reference strain P155. Of note, this *blpC* type was associated exclusively with non-encapsulated pneumococci.

Looking at the sets of co-colonization samples, 40.6% of the dual interactions were between strains of the same BlpC type, while 59.4% were between strains of different BlpC types. When the frequency in which BlpC types appear together in co-colonization was compared with the frequency estimated under the hypothesis that strains mix randomly no significant difference was found (p=0.577). The same analysis was performed considering only pairs of strains in which secretion of BlpC was confirmed phenotypically through overlay assays in one of the strains and the results were the same (p=0.798).

Of note, a permutation analysis showed that strains of *blpC*$_{6A}$ are found with strains of *blpC*$_{T4}$ at a higher frequency than the one estimated based on the frequency of these two types in the collection (p=0.04).

High prevalence of strains with disrupted *blpA* gene co-colonizing in nature. Disruption of the *blpA* transporter gene has been previously associated to a *cheater*, i.e., non-inhibitory immune-only, phenotype (Son *et al.*, 2011). Analysis the *blpA* gene confirmed a disruption in the gene in 155 out of 285 strains (54.4%). Of these, 151

strains contained the 4 bp repeat insertion described by Son *et al.* (Son *et al.*, 2011) and 4 strains presented a larger deletion, as confirmed by the amplification of the total *blpA* gene.

The presence of these frameshift-causing insertions and deletions was an explanation for the high proportion of strains with a non-inducible locus in the signaling overlay assays, as 69% of the non-inducible strains were in fact *cheaters*. *blpA* disruption was mainly associated with strains of type $blpC_{R6}$ and $blpC_{T4}$ (p=0.0001 for both, Fisher's exact test) (Figure 2).

In the sets of co-colonized samples, 27.9% of dual interactions occurred between two *cheater* strains, 20.6% between strains with an intact *blpA* gene (non-*cheater*), and 51.5% of interactions occurred between a *cheater* and a non-*cheater* strain. Comparison of the estimated and observed proportions of events involving strains with same or different *blpA* status (intact or disrupted) did not show a significant difference, suggesting that the *cheater* phenotype alone does not restrict co-colonization of pneumococci (p=0.713).

Phenotypes of bacteriocin secretion do not restrict co-colonization. To determine the phenotype of bacteriocin secretion, *i. e.*, the inhibitory activity of the pneumococcal strains, overlay assays with a susceptible reporter strain P537 (Δ*blp*) were performed.

Eighty-four out of 285 (29.5%) co-colonizing strains displayed inhibitory activity against P537. Of these, 21 strains (25%) had a disrupted *blpA* gene, suggesting a non-*blp* mediated inhibition. The remaining 63 strains had an intact *blpA* gene. Of these, we were able to confirm corresponding BlpC pheromone secretion with the signaling overlays in 63.1% (n=53) of inhibitory strains and failed to do so in 11.9% (n=10).

Among the strains that did not display inhibitory activity (n=201), 67% were *cheaters* and 30% did not secrete pheromone in the signaling overlays, despite an apparently

intact *blpA* gene. Six strains had a functional locus confirmed by the signaling overlays but still failed to inhibit the susceptible strain P537. The BIR prediction of these six strains revealed the presence of several bacteriocin peptides, including *blpI*, *blpJ*, *blpK*, *blpM*, and *blpN*, which have been shown to function as inhibitory peptides (Dawid *et al.*, 2007; Lux *et al.*, 2007; Son *et al.*, 2011). Of note, none of these strains were able to inhibit any of the control strains with known BIR content (Table 1) in inhibition overlay assays, contrary to what would be expected based on genetic content of the BIR, *blpA* and *blpC*.

In the sets of co-colonized samples, co-existence of strains that displayed an inhibitory phenotype with was not different from the expected by chance, suggesting that an inhibitory phenotype alone does not prevent co-colonization (p=0.715).

Considering this result, we performed an *in silico* prediction of the outcome of the interaction of each pair of strains in every sample, taking into account the genetic content of the BIR, the integrity of the *blpA* transporter gene and the type of signaling peptide BlpC secreted. Our prediction resulted in 50 outcomes of inhibition (30.3%) and 115 outcomes of no inhibition (69.7%). Comparison of the estimated and observed proportions of outcomes of inhibition and no inhibition did not show a significant difference (p=0.274).

The results obtained with the genotypic and phenotypic approaches suggest that the *blp* locus alone does not seem to prevent the co-existence of pneumococci in the host.

Phenotypes of bacteriocin secretion are the same in strains isolated from single and co-colonization events. In order to assess whether strains found in single and co-colonization events would display different genotypic and/or phenotypic *blp* characteristics, we selected for comparison a subset (n=298) of the initial collection of 1,053 nasopharyngeal samples screened for carriage of multiple serotypes in which only one strain was detected. This selection was performed randomly but maintaining a

matched number of samples from each year between both collections. The characteristics of this collection are summarized in Table S2.

Overall, the prevalence of each *blpC* type was similar in the two collections, as well as the distribution of inducible, non-inducible and *cheater* strains within each *blpC* type (Figure 2 and 3A).

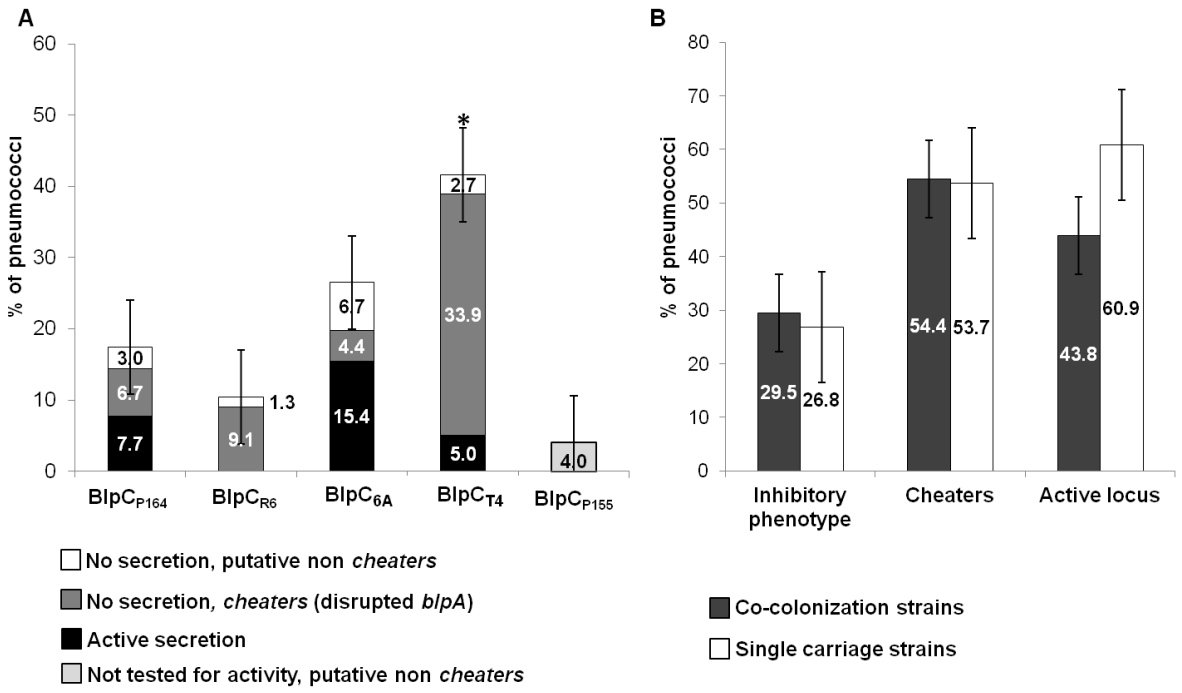

Figure 3. Characterization of the blp locus of the strains isolated from single carriage events. (A) Distribution of *blpC* types in the co-colonization strains. Asterisk indicates statistically significant differences. (B) Comparison of the single and co-colonized collections regarding proportions of strains with inhibitory phenotype, cheater genotype and locus inducibility. Error bars represent standard error.

The proportion of strains isolated from single carriage that displayed an inhibitory phenotype against the susceptible strain P537 was comparable to the one observed in the co-colonization strains (26.8% and 29.5%, respectively). The same was observed for the proportion of strains with a disrupted *blpA* gene (53.7% and 54.4%, respectively) and the proportion of strains with evidence of pheromone secretion revealed by the β-galactosidase activity in the signaling overlays (60.9% and 43.8%, respectively) (Figure 3B).

Comparison of the results of the two collections suggests that pneumococci that are found in single and co-colonization events seem to possess the same phenotypes of bacteriocin secretion, corroborating that, with the approach used in this study, the *blp* locus seems to have little impact on the co-existence of strains in the nasopharynx.

Discussion

In this study we have identified a collection of co-colonized nasopharyngeal samples to assess the impact of the *blp* locus and bacteriocin secretion on the co-existence of pneumococci in the host. We have also identified a matched collection of pneumococci isolated from single carriage events that was used as a reference to evaluate possible differences between strains in single and co-colonization.

Characterization of the co-colonization strains at the level of serotype and genotype enabled us to identify positive associations between specific capsular types and genotypes, although no straightforward observations could be found to explain those associations. At the capsular level, we explored properties shown to be dependent on the capsular type, such as the polysaccharide structure, and, by association, the fitness cost of capsule production (Hathaway *et al.*, 2012; Weinberger *et al.*, 2009), and the surface charge of the capsular type (Li *et al.*, 2013). All these properties have been shown to predict to the prevalence of the serotypes. On one hand it was proposed that serotypes with capsule structures with more carbon in their polysaccharide repeat unit would require more energy for capsule production and would have smaller capsules. Therefore, these serotypes would be less resistant to phagocytosis and clearance, which could explain their low prevalence (Hathaway *et al.*, 2012; Weinberger *et al.*, 2009). On the other hand, the surface charge was also proposed to explain the prevalence of serotypes, since capsules more negatively charged were shown to repel host immune cells that contribute to clearance, such as neutrophils and macrophages

(Li *et al.*, 2013). We assessed all these properties in our collection of co-colonized samples: the number of carbon molecules per polysaccharide repeat unit was determined based on the published capsule structures (Bentley *et al.*, 2006) and the surface charge was inferred from the data published by Li *et al*. (Li *et al.*, 2013). Our prevalence results are in agreement with the proposed hypothesis but none of the explored properties could be used to predict capsular interactions (data not shown).

The genetic characterization of the *blp*-locus of the co-colonizing strains allowed us to show a very large diversity in this locus, originated from the diversity in the *blpC*, *blpA* and BIR region, supporting observations from other studies (Bogaardt *et al.*, 2015; Dawid *et al.*, 2007; de Saizieu *et al.*, 2000; Lux *et al.*, 2007; Son *et al.*, 2011). Notwithstanding, MLST genotyping of the strains enabled us to show a fairly high level of conservation of the *blp*-locus among closely-related strains.

Overall our genotypic and phenotypic characterization of the *blp*-locus of co-colonizing pneumococci seems to suggest that the phenotypes of bacteriocin secretion do not have an impact in the co-existence of pneumococci in the nasopharynx, an observation that was supported by the lack of differences in the results obtained in the characterization of the single carriage isolates. Given the large diversity in this locus, it is not surprising that the effect of *blp*-mediated competition on co-colonization is not as straightforward as might be expected and several aspects must be taken into account. First, the high diversity of BlpH receptors, that can be higher than the diversity of BlpC peptides due to the existence of naturally occurring chimeras for the $blpH_{6A}$ gene, as described by Pinchas *et al.* (Pinchas *et al.*, 2015), and may affect the likelihood of cross stimulation between co-colonizing pairs. These authors have shown that these BlpH variants have different specificities for cognate and non-cognate BlpC peptides, which can have an effect in the race for *blp* locus activation. Pinchas *et al*. have also shown some level of specificity of $BlpH_{T4}$ to the non-cognate signalling peptide $BlpC_{6A}$ and have proposed that this ability to respond to pheromone secreted by other strains

might act as a compensation for the fact that most of $BlpH_{T4}$ have a disrupted *blpA*. Our colonization data further supports this hypothesis, as we confirmed that $BlpC_{T4}$ strains are the most prevalent BlpC type and are mostly *cheaters*. Furthermore, we have found a positive association that was observed between strains of $blpC_{6A}$ and $blpC_{T4}$ types that might be explained by the promiscuity of the BlpH receptor. Second, the large array of bacteriocins and immunity proteins that can be present in a strain, and the fact that the pneumococcal natural niche is such an hostile polymicrobial environment, raise the hypothesis that this competition mechanism might be used for mediating the interaction not only with bacteria from the same species, but also with other inhabitants of the nasopharynx. In fact, Lux *et al.* (Lux *et al.*, 2007) have demonstrated the inhibitory activity of pneumococci against bacteria of different species. Finally, the natural and highly frequent occurrence of strains that display a *cheater* phenotype also contributes to the complexity of this competition mechanism. The reason why these cheater strains are so highly prevalent remains to be addressed and the advantage of displaying this phenotype is not completely clear. On one hand, these strains avoid the fitness-cost of bacteriocin secretion while they are still able to express immunity proteins. On the other hand they become at risk of elimination upon the encounter of a strain secreting a different BlpC type. Recently Kjos *et al.* (Kjos *et al.*, 2015) have suggested a connection between the *com* and *blp* systems, demonstrating that a strain with a disrupted *blpA* (D39) was able to activate its *blp* locus using *comAB* as a replacement transport system when competence is activated. We have performed signalling overlays in close to 600 strains, from which over half had a disrupted *blpA* gene, and we were never able to see locus activation in a strain with *blpA* disruption. Nevertheless, we have repeated the analysis performed in this study considering that all strains would be able to activate the *blp* locus and secrete bacteriocins and the conclusion that bacteriocin secretion does not restrict co-colonization was maintained.

Our study has some limitations. First, we did not sequence individual genes in the locus. Given this, the strains that had apparently intact *blpA* genes but lacked pheromone secretion may have been inactive due to uncharacterized mutations in *blpA, blpH* or *blpR*. Another explanation could be a lack of sensitivity of the assay or particular conditions of locus activation in those strains. In addition, the few non-inhibitory strains with intact pheromone secretion may have harbored mutations in their bacteriocin genes that would not be detected with the RFLP analysis. Second, the cross-sectional nature of our study prevented us from measuring the effect of *blp*-mediated competition on the duration of carriage. Also, we did not measure the pneumococcal load in the samples and it is possible that the effects of competition might be reflected at the level of carriage density. Even so, we measured the relative proportion of each strain in the samples and could not establish a correlation between phenotypes of bacteriocin secretion and strain density. The third limitation is the fact that we did not measure the ability of the strains to activate the *blp* locus in vivo, which would be a requirement for this mechanism to have an impact on co-colonization. Nevertheless, Dawid *et al*. (Dawid *et al.*, 2007) have used wild type and bacteriocin-deleted mutants in dual colonization experiments and have observed that the production of bacteriocins by the wild type strains was able to reduce colonization with the deleted mutants. Also, Son *et al.* (Son *et al.*, 2011) have also shown a competitive advantage of baceriocin producers vs sensitive, compared with bacteriocin producers vs immune strains. These results showed that the locus is active *in vivo* and is able to play a role in the polymicrobial environment of the nasopharynx.

Our study also has significant strengths. We have used a very well characterized, natural collection of co- and singly colonized samples identified through the use of highly sensitive molecular methods, which enabled us to obtain an unbiased and highly diverse collection of both single and co-colonizing pneumococcal strains. To our

knowledge, this is the first time that co-colonized samples have been used to measure the impact of *blp*-bacteriocin production on competition in the nasopharynx.

Although we have not seen an effect of *blp*-mediated competition on co-colonization, the fact that this locus is present in all pneumococci and has been maintained by evolution (Bogaardt *et al.*, 2015) suggests an important function, which is supported by the results obtained in competition experiments showing that the locus is active *in vivo* (Dawid *et al.*, 2007). Exactly what this function is and at which level it is exerted remains to be fully elucidated. It could be important under specific conditions (*e.g.*, nutrient limitation) or during the establishment of a strain during colonization. Also, studies with a longitudinal design would help to determine whether *blp*-mediated competition might be acting at the level of carriage duration or density.

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Supplementary material

Table S1. Primers designed for assignment of *blpC* types and specific amplification conditions.

Primer name	*blpC* type[a]	Primer sequence (5'-3')	Fragment size (bp)	[$MgCl_2$] (mM)	Annealing (ºC)
blpC_up	all	GGATAAGAAACAAAACC			
blpC-I_dn	*blpC*$_{T4}$	GTGATGTAATGAGCATATC	123	2	52
blpC-IIa_dn	*blpC*$_{R6}$	GCTGAATAGGTAGTTCAAGTGC	149	3	58
blpC-IIb_dn	*blpC*$_{P164}$	GGCTGATAAAATCC	141	3	56
blpC-IIc_dn	*blpC*$_{6A}$	GTCCTTTCGTATTGTTATGC	131	2	56
blpC-IId_dn	*blpC*$_{P155}$	GCACTGGTTGATTAGATCC	146	2	50

[a] Nomenclature used by Son *et al.(Son*

Table S2. Genetic characteristics of the collection of isolates from single carriage events

Serotype (no. isolates)	**CSP type**	***blpC* types**	***blpA***
3 (13)	CSP1	$blpC_{R6,}$ $blpC_{6A}$, $blpC_{T4}$	Disrupted/Intact
6A (23)	CSP1, CSP2	$blpC_{P164}$, $blpC_{6A}$, $blpC_{T4}$	Disrupted/Intact
6C (30)	CSP1, CSP2	$blpC_{P164}$, $blpC_{R6}$, $blpC_{6A}$	Disrupted/Intact
11A/D (24)	CSP1	$blpC_{P164}$, $blpC_{6A}$	Disrupted/Intact
15A (17)	CSP1	$blpC_{T4}$	Disrupted/Intact
15B/C (28)	CSP1	$blpC_{R6}$, $blpC_{T4}$	Disrupted
19A (61)	CSP1, CSP2	$blpC_{6A}$, $blpC_{T4}$	Disrupted/Intact
19F (35)	CSP1, CSP2	$blpC_{P164}$, $blpC_{R6}$, $blpC_{6A}$	Disrupted/Intact
21 (14)	CSP1	$blpC_{P164}$, $blpC_{T4}$	Disrupted
23A (15)	CSP1	$blpC_{R6}$, $blpC_{6A}$	Disrupted/Intact
23B (25)	CSP1	$blpC_{P164}$, $blpC_{T4}$	Disrupted/Intact
NT (13)	CSP1	$blpC_{T4}$, $blpC_{P155}$	Intact

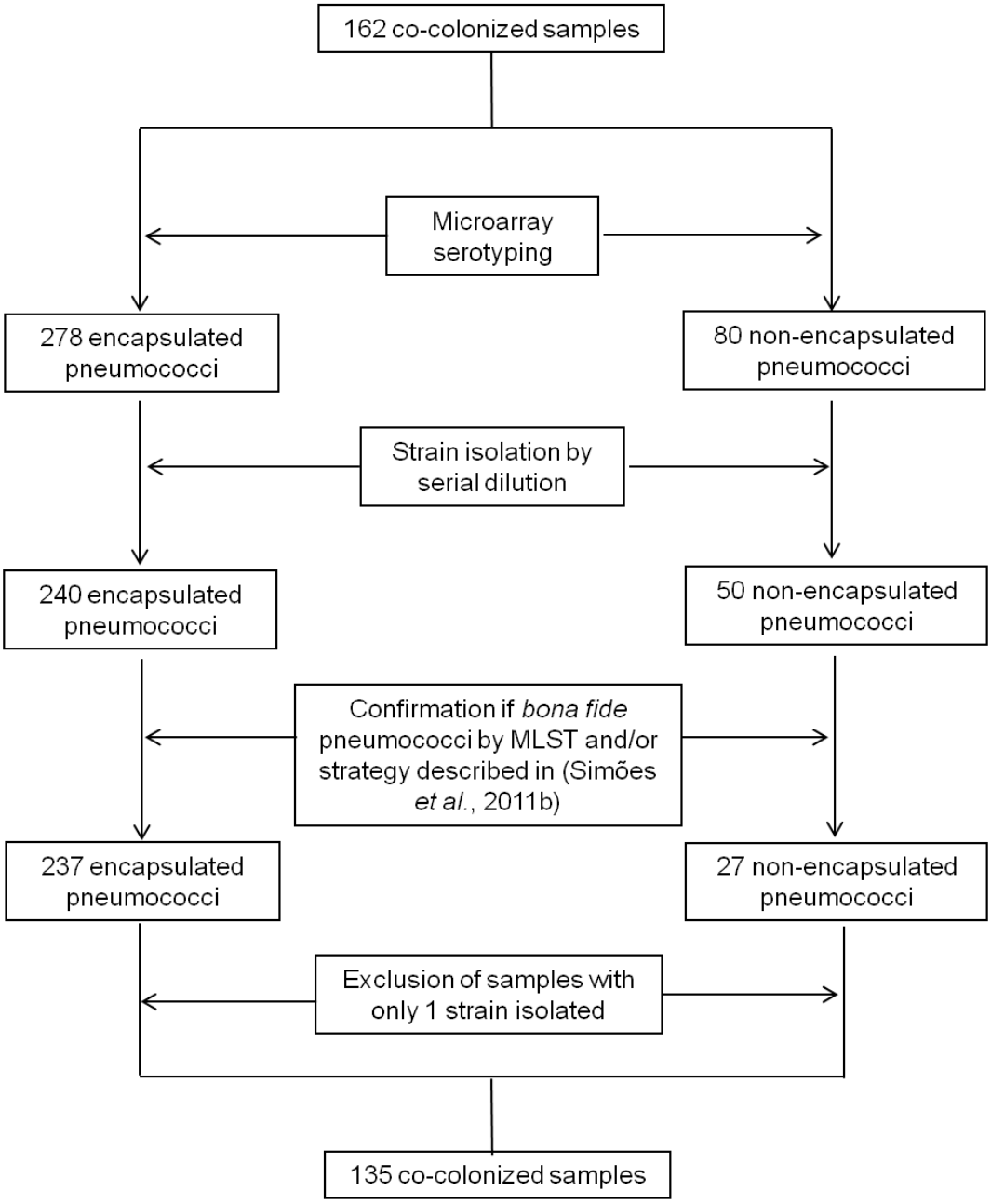

Figure S1. Summary of results of the strategy used for isolation of pneumococcal strains identified by the capsular microarray in the co-colonized samples.

Simões, A. S., Valente, C., de Lencastre, H. & Sá-Leão, R. (2011b). Rapid identification of noncapsulated *Streptococcus pneumoniae* in nasopharyngeal samples allowing detection of co-colonization and reevaluation of prevalence. *Diagn Microbiol Infect Dis* **71**, 208-216.

Chapter VII

Concluding remarks

Pneumococcal co-colonization of the nasopharynx has been recognized for several years but its dynamics, prevalence and effects on pneumococcal population have remained largely unknown. The work developed under the scope of this thesis enabled us to determine the prevalence of co-colonization among Portuguese children and to evaluate the impact of the introduction of pneumococcal conjugate vaccines on its dynamics. Moreover, using an epidemiological approach, we have explored molecular mechanisms that could influence co-colonization, focusing on bacterial-related properties such as the capsule, the genetic background, competence mediated-fratricide, and bacteriocin secretion.

The results presented in **Chapter II** show that vaccination with PCV7 resulted in a lower co-colonization rate due to an asymmetric distribution between NVTs found in single and co-colonized samples. We propose that this asymmetric distribution is attributable to the fact that some NVTs prevalent in the PCV7 era might be more competitive than others, hampering their co-existence in the same niche. This result may have important implications since a decrease in co-colonization events is expected to translate in decreased opportunities for horizontal gene transfer, hindering pneumococcal evolution events such as acquisition of antibiotic resistance determinants or capsular switch, which might represent a novel potential benefit of conjugate vaccines.

As a follow up of the study presented in **Chapter II**, the study presented in **Chapter III** was conducted. This study had a similar design to allow comparisons but was conducted after the introduction of PCV13 and was aimed at evaluating the effect of this vaccine on pneumococcal co-colonization. Despite some differences in the methodological approaches the conclusions of both studies were similar. In **Chapter III** we also showed that PCV13 vaccinated children are less co-colonized than non-vaccinated children from the same era. As in the PCV7 era, we observe an asymmetric redistribution of NVTs in single and co-colonization events. This observation supports

our previous hypothesis of different competitive abilities among NVT serotypes, which prevents their co-existence.

The results presented in **Chapter III** show also that PCV13 serotypes are still prevalent in a population with no universal but very high vaccine coverage, mainly among non-vaccinated children. While in vaccinated children PCV13 serotypes are infrequent and found mainly as a minor serotype, in non-vaccinated children these serotypes are still highly prevalent and in high abundance.

Finally, the results of **Chapter III** show also a very high prevalence of non-encapsulated pneumococci and *Streptococcus spp*., particularly among vaccinated children. Whether it is an effect of the use of conjugate vaccines and which role these species can play in the nasopharyngeal ecosystem and in the evolution of pneumococci are questions that remain to be addressed.

Still in the context of assessing the prevalence of co-colonization, in **Chapter IV** it is shown that the use of methodologies based on colony morphology to detect co-colonization events results in an overrepresentation of serotypes that display very distinctive colony morphologies, such as serotype 3 and non-encapsulated pneumococci, introducing a significant bias in the estimated serotype distribution of the pneumococcal population.

In **Chapters V** and **VI** we explored molecular mechanisms that could potentially have an impact on the co-existence of pneumococci in the nasopharynx.

In **Chapter V** a study focused on the impact of pherotype (or competence stimulating peptide) on pneumococcal nasopharyngeal co-colonization was conducted. Despite the bias that might be introduced in the collection by the fact that co-colonized samples were identified based on colony morphology, this study showed that pneumococcal pherotypes co-colonize individuals independently, suggesting that pherotype-mediated fratricide is not an important mechanism of within-host competition. Our results are in

agreement with a previous study of similar design but in a different setting (Vestrheim *et al.*, 2011), which supports our findings. Also, the same type of analysis was performed in the collection presented in **Chapter VI**, obtained through the use of highly sensitive methods for systematic detection of co-colonization, and the results were the same.

In **Chapter VI** we explored the role of the *blp* locus and bacteriocin secretion as a competition mechanism that could potentially influence the co-existence of pneumococci in the host. As already mentioned, we have used a collection of co-colonized samples identified through the use of molecular methods that enabled us the systematic detection of co-colonization events. As such, we have obtained a highly diverse collection of co-colonizing isolates that allowed us to explore the possible existence of associations at the level of serotype and genotype. In fact, we were able to find positive associations between specific capsular types and genotypes, although none of the explored properties could explain such associations.

As for the impact of the *blp* locus on co-colonization, we show in **Chapter VI** that co-colonizing pneumococci present high genetic diversity at the level of this locus and display several different phenotypes of bacteriocin secretion and immunity. We show also that pneumococcal strains co-colonize individuals independently of the genetic content of their *blp*-locus and independently of their phenotypes of bacteriocin secretion. In addition, we demonstrate that pneumococci identified in single carriage events are similar to co-colonizing pneumococci regarding the genetic characterization of the *blp* locus and the phenotypes of bacteriocin secretion.

The work developed under the scope of this thesis gives an important contribution to the available knowledge on co-colonization. Nevertheless, important questions remain to be addressed.

The first question is related to the major limitation of our studies. Because we have used an epidemiological approach based on cross-sectional studies, we were not able to measure if the bacterial properties and molecular mechanisms that we have explored to explain co-colonization somehow might be affecting the density and duration of carriage events or the carriage of a specific strain that could be at a disadvantage. Several epidemiological and experimental observations point to the fact that this might be a possibility, as presented in **Chapter I**. Studies with a longitudinal design are necessary to determine whether the explored properties and competition mechanisms might be acting at the level of carriage duration or density. Also, we have not addressed if the two competition mechanisms explored in the context of this thesis (pherotype-mediated fratricide and bacteriocin secretion) are active and relevant in an *in vivo* context.

The second question concerns the possible effect of the microbiota on pneumococcal intra-specific interactions, which was not taken into consideration in these studies. The nasopharynx, however, is a complex polymicrobial environment and there is increasing evidence that the pneumococcus is involved in multispecies biofilms (Hall-Stoodley *et al.*, 2006) (for a revision see (Tikhomirova & Kidd, 2013)). 16S rRNA-based microbiome studies have revealed high variability in nasopharyngeal microbial communities across individuals and possibly across seasons of the year (Bogaert *et al.*, 2011; Laufer *et al.*, 2011). Epidemiological studies have shown a positive association between *S. pneumoniae* and two other pathogenic inhabitants of the nasopharynx, *Haemophilus influenzae* and *Moraxella catarrhalis* (Jacoby *et al.*, 2007; Mackenzie *et al.*, 2010; Pettigrew *et al.*, 2008), although experimental data is not in agreement with this observation. A study conducted in a neonatal rat model showed that established colonization by *S. pneumoniae* facilitated colonization by *H. influenzae* (Margolis *et al.*, 2010) but it was also shown in an in vitro study that the hydrogen peroxide produced by *S. pneumoniae* inhibits the growth of *H. influenzae* and *M.*

catarrhalis (Pericone *et al.*, 2000). Also, Lysenko *et al*. (Lysenko *et al.*, 2005) showed that *H. influenzae* was dominant in a mouse model through the stimulation of opsonophagocytic killing of *S. pneumoniae*. In addition, there is increasing evidence that bacteria growing together in the nasopharynx can alter one another's gene expression (Cope *et al.*, 2011) but these mechanisms of interspecies gene regulation remain poorly elucidated. The quorum sensing system (QS) LuxS/autoinducer-2 (AI-2) has been shown to mediate interspecies communication and has the potential to influence virulence and growth, as reviewed in (Pereira *et al.*, 2012). In pneumococci, it was shown that LuxS-deficient strains are unable to form biofilms at early time points and have impaired DNA release ability (Trappetti *et al.*, 2011; Vidal *et al.*, 2011). Moreover, it was also shown that the LuxS/AI-2 QS system is involved in the persistence of pneumococci in the nasopharynx (Joyce *et al.*, 2004) and in the persistence of *H. influenza* biofilms in the ear cavity (Armbruster *et al.*, 2009). Overall, these data point to the fact that intraspecific interactions in the context of a polymicrobial environment might be modulated by interactions with other species and should not be disregarded.

The third question is the role of bacteriophage predation, which has been shown to be frequent among pneumococci (Ramirez *et al.*, 1999), on the interaction and co-existence of pneumococcal strains in the nasopharynx. Phage predation has been shown to drive evolution (Weinbauer & Rassoulzadegan, 2004) and to induce genetic plasticity that can contribute to the adaptability of pneumococci inside the host. More importantly, phages can increase the fitness of the bacterial host, namely by encoding virulence factors that contribute to bacterial survival in the mammalian host (Brussow *et al.*, 2004). Moreover, Carrolo *et al.* (Carrolo *et al.*, 2010) have shown that prophage spontaneous activation enhances pneumococcal biofilm formation by increasing the release of DNA into the extracellular medium and Loeffler *et al.* (Loeffler & Fischetti, 2006) have shown that prophage activation increases adherence ability of pneumococci to human epithelial cells, both important factors to promote colonization.

On the other hand, the large array of phage resistance mechanisms used by bacteria indicate that prophage carriage must have an associated fitness cost to the bacteria (Labrie *et al.*, 2010). Not many studies have been performed to address this question but DeBardeleben et al. (DeBardeleben *et al.*, 2014) have shown that a strain carrying a prophage element was outcompeted by its isogenic non-infected sister strain, indicating that carriage of that prophage element decreases the fitness of a strain during colonization.

The fourth question is related to the effect of the competition mechanisms explored in the context of this thesis on the biofilm structure, as it is generally recognized that this lifestyle is important for colonization (Hoa *et al.*, 2009; Kania *et al.*, 2008; Marks *et al.*, 2012; Psaltis *et al.*, 2007; Sanclement *et al.*, 2005). Carrolo *et al.* (Carrolo *et al.*, 2014) have shown that the presence of CSP in the medium increases the ability of strains to form biofilms and that intra-pherotype genetic exchange prevails in these conditions, probably due to the spatial organization of the strains in the biofilm. Also, there is evidence in the literature that some competition mechanisms are used by bacteria to conquer their space within the community, highlighting that positioning within the microbial community is important for survival (Kim *et al.*, 2014). Additionally, Oliveira et al. (Oliveira *et al.*, 2015) have recently changed the paradigm on the relationship between biofilm formation and ecological interactions among bacteria, by proposing that biofilm formation is a response to bacterial competition rather than a cooperative behavior. The strategy used in this thesis would not allow us to measure the effect of fratricide or bacteriocin secretion at this level, but given that evolution has maintained these competition mechanisms, it is possible that their effect might be exerted at a smaller scale than the one measured here.

Finally, but not less important, the fifth question concerns the effect of the host immune response on colonization and how it could affect carriage of multiple strains and interactions between strains. Brugger *et al.* (Brugger *et al.*, 2010) showed that

colonization density was significantly higher in nasopharyngeal swabs containing multiple pneumococcal strains, compared to swabs with a single strain. Also, Margolis *et al.* (Margolis *et al.*, 2010) have shown that sequential colonization with two pneumococcal strains results in increased colonization densities to allow the co-existence of the pulsed and the established strains. Both these studies suggest that carriage of multiple strains result in higher carriage densities. The effect of this higher density in the host immune response remains to be addressed but could ultimately have an impact on pneumococcal carriage. Supporting this hypothesis is the fact the immune modulation induced by the presence of other species results in different clearance rates of pneumococci (Lysenko *et al.*, 2005). Accordingly, possible changes in the immune response induced by carriage of multiple serotypes could have a similar effect. Trzciński et al. (Trzcinski *et al.*, 2015) have recently shown in co-infection experiments in a mouse model that the innate immunity of naïve mice does not influence the relative rankings of serotypes, suggesting no effect on serotype interactions in the host. Nevertheless, the same study has shown that, although no changes were found in the competitive relationships between serotypes, non-capsular immunity induced by previous mice immunization resulted in lower total CFU counts recovered from mice, suggesting an indirect effect of acquired immunity on carriage of multiple strains.

The results obtained in the context of this thesis do not allow us to exclude the possibility that pneumococci do not compete when in co-colonization. In fact, the already mentioned observation of Margolis et al. (Margolis *et al.*, 2010) that sequential colonization with two pneumococcal strains resulted in increased colonization densities to allow the co-existence of the both strains points towards the idea of co-existence rather than competition between pneumococcal strains in the nasopharynx. Moreover, if in fact the ecological principles can be transposed to the microbial world, the principle that similar competitive abilities facilitate co-existence, proposed by Bengtsson *et al.*

(Bengtsson *et al.*, 1994), supports this hypothesis. On the other hand, the array of direct and indirect competition mechanisms maintained by evolution in the pneumococcus (Dawid *et al.*, 2007; Guiral *et al.*, 2005; Lux *et al.*, 2007; Nelson *et al.*, 2007) seem to suggest that competition does occur, which is supported by epidemiological and experimental studies (Auranen *et al.*, 2009; Lipsitch *et al.*, 2000). Also, studies with other microbial species further support this hypothesis of competition rather than cooperation between microbes in the same niche (Foster & Bell, 2012).

Overall, the studies presented in this thesis give an important contribution to a poorly explored field. We showed that co-colonization is a frequent phenomenon and how it can be affected by the use of conjugate vaccines and by certain mechanisms intrinsic to pneumococcal biology. Still, many interesting questions remain to be answered and should be the focus of further research studies.

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