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**“Cost effective manufacturing methods for Public Health vaccines
against Gram negative bacteria”**

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I. SUMMARY

Infectious diseases, particularly in less developed countries, remain among the leading causes of global mortality, most of these infections are caused by Gram-negative bacteria which are the major pathogens responsible of neonatal sepsis and cause of a range of diarrheal, respiratory and invasive diseases.

The most frequent gram-negative isolates are *E.coli*, *Salmonella* spp and *Shigella* spp.

Salmonella infection represents a considerable burden in both developing and developed countries. Efforts to reduce transmission of salmonellae by food and other routes must be implemented on a global scale thus the development of cost effective, needle-independent vaccines is a high priority.

Vaccine for Developing Countries should be designed to be inexpensive (less than one euro for association like UNICEF and GAVI), stable at room temperature, simple to administer to infants, readily administered by nonprofessionals using a needle-free method and the vaccines formulation would protect against multiple agents.

Novartis Vaccines Institute for Global Health (NVGH) aims to develop a broadly protective vaccine using outer membrane particles. These particles, called Generalized Modules for Membrane Antigens (GMMA), also known as outer membrane vesicles (OMV), are naturally shed from the surface of living Gram-negative bacteria. GMMA are different from the detergent-extracted OMV which are depleted of lipooligosaccharides and lipoproteins.

The purpose of this PhD project was to investigate the possibility to develop low cost vaccines applicable to Gram-negative strains, intended for neglected infectious diseases of impoverished communities, using the GMMA technology platform.

This work showed the possibility to use GMMA particles as starting material for membrane components extraction, optimizing new and innovative platform, particularly for LPS or proteins recovery, in order to decrease the costs of goods (COGs).

The present work is focused on the O: 4,5-antigen polysaccharide extraction (using the acid hydrolysis approach), purification and characterization from *Salmonella* Typhimurium SL1344 *tolR*⁻ GMMA.

NVGH, to over produce and collect GMMA, is developing new methods using over producing GMMA mutant strain (the *tolR* gene deletion results in substantial overproduction of outer membrane blebs) and two consecutive TFF steps (Tangential Flow Filtration steps: micro-filtration at 0.2 µm and thereafter a second micro-filtration at 0.1 µm) at the end of the fermentation process to collect GMMA from bacterial suspensions.

NVGH has developed another innovative platform for O-antigen extraction from biomass at the end of the fermentation process, using the acid hydrolysis approach of whole bacterial culture.

The present work shows for the first time the possibility to use GMMA particles as O-antigens source (and in general as source of membrane components) due their membrane composition enriched in LPS. We have proven that, O-antigens can be extracted from GMMA using the direct acid hydrolysis method thus the consequent O-antigen purification process is easy and cost effective.

This work provides the basis for the development of new vaccines, glyconjugate or proteins based, exploiting the same approach used and developed for *S. Tyhphimurium* SL1344 *tolR*⁻ GMMA, which could be applied to other Gram negative strains.

II. INTRODUCTION

1. ENTERIC DISEASES IN DEVELOPING COUNTRIES

Every year 3·3 million babies die within the first 28 days of life (the newborn or neonatal period); newborn deaths account for 41% of all child deaths in developing countries. Another million babies are stillborn. Effective interventions could prevent most of these deaths. The challenge is to identify strategies that can be implemented feasibly in the short term to ensure that newborn babies have access to these life-saving interventions [1,2].

Infectious diseases, particularly in less developed countries, remain among the leading causes of global mortality, exceeding 60% in children up to 4 years old [3].

Poor sanitary conditions, unsatisfactory traditional practices concerning waste disposal and poor personal hygiene in general, facilitate the spread of enteric pathogens [4].

Only washing hands with soap could be possible reduce the risk of diarrheal diseases by 42–47% and interventions to promote hand-washing might save a million lives [5], but no radical improvements of these conditions can be expected in most developing countries in the near future. Thus, vaccines become very important in the multi-faceted approach necessary to control diarrheal diseases either through protection or treatment [6].

In addition to the acute morbidity and mortality associated with enteric diseases, diarrhea is a cause and effect of malnutrition, which may be one of the greatest yet of the “emerging infectious diseases” (Bill and Melinda Gates Foundation, Enteric and Diarrheal Diseases, strategy overview) [7].

The consequences of early childhood diarrhea on growth and also on physical and cognitive development lead to tremendous losses of human potential and productivity [8].

Enteric diseases are also a significant cause of morbidity among people from more developed countries. In the United States, for example, diarrhea is one of the most frequent reasons given for visits to the pediatrician’s office and it is also a major threat to travelers [9].

Malnutrition remains the major adverse prognostic indicator for diarrhea related mortality [10]. The wide diversity of bacterial and viral infections that may cause diarrhea [11] complicates accurate surveillance and diagnosis especially in developing countries with little or no access to modern laboratory procedures. The specific disease burden attributable to particular

infectious antigens is particularly complex, given the multiplicity of these antigens and their serotypes, and its accurate documentation depends largely on laboratory facilities.

Gram-negative are major pathogens of neonatal sepsis in developing countries, these bacteria causing a range of diarrheal, respiratory and invasive diseases [12].

The most frequent gram-negative isolates are *E.coli*, *Salmonella* spp and *Shigella* spp, these include pathogens that are non-invasive, such as *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC); pathogens that produce gut inflammation, such as *Shigella* and rotavirus; and pathogens that invade through the intestine and enter the systemic circulation, such as *Salmonella typhi*, polio virus, and human immunodeficiency virus [13].

Preventive strategies on a global scale might ultimately hold the greatest potential to reduce the burden of diarrhoeal disease. These strategies include vaccines and, most importantly, policies to address persisting inequalities between the developed and developing countries [10]. While, in the long term, access to clean water, better hygiene, adequate nutrition, and improvement of sanitary measures would certainly have the greatest impact on diarrheal diseases [12].

2. CURRENT STATUS OF VACCINES DEVELOPMENT

Infectious diseases remain a major cause of deaths and disabilities in the world, the majority of which are caused by bacteria. Although immunization is the most cost effective and efficient means to control microbial diseases, vaccines are not yet available to prevent many major bacterial infections. Examples include dysentery (shigellosis), gonorrhoea, trachoma, gastric ulcers and cancer by *Helicobacter pylori*. Improved vaccines are needed to combat some diseases for which current vaccines are inadequate [14] thus the modern biotechnology is allowing the development of new vaccines against diseases that were previously unassailable [15].

Enteric infections resulting in diarrheal disease remain a leading global health problem. Acute diarrheal disease may be caused by several infectious agents and the relative contribution of any specific agent requires laboratory identification of the agent from patients [16] [14,17].

The establishment of the germ theory and the identification of specific microbes as the causative agent of a wide variety of infectious diseases led to enormous progress, notably the

development of vaccines and ultimately of antimicrobials. Research efforts focus on the development of vaccines and new drugs, and on re-establishing public health measures [18].

93.8 million Cases of gastroenteritis due to *Salmonella* species occur globally each year, with 155,000 deaths. *Salmonella* infection represents a considerable burden in both developing and developed countries. Efforts to reduce transmission of salmonellae by food and other routes must be implemented on a global scale [19] thus the development of cost effective, needle-independent vaccines is a high priority [20].

The history of vaccine development can be marked by a number of milestones resulting from revolutions in technology. Earlier waves involved pathogen attenuation, inactivation, and viral cell

culture, all supported by an increased understanding of the human immune response [21]. However, recombinant DNA technology has probably been the single most innovative advance that has opened the door to new technological developments.

A. New vaccines technological development

a) Recombinant DNA Technology

Recombinant DNA technology was first successfully applied to vaccines over 25 years ago, with the production of a recombinant vaccine for Hepatitis B virus (HBV) [22]. Along with the need for developing better-characterized, less reactogenic or more potent vaccines to replace the existing vaccines, a more recent driver for developing recombinant subunit proteins has been the need to provide broader protection against multiple strains or serotypes of a bacterium (e.g., serogroup B *meningococcus*, *pneumococcus*).

Strategies to increase the breadth of vaccine coverage include the engineering of multiple epitope based vaccines, incorporating epitopes either from different antigens of the same pathogen or even from different pathogens into a single protein using synthesized genes [22].

Another major use of recombinant DNA technology has been in site-specific inactivation of toxins as a safe and efficient alternative to chemical inactivation. The use of recombinant DNA has been applied to the genetic attenuation of bacteria and viruses for use in live attenuated vaccines. Previously, attenuation of bacteria or viruses was achieved through serial

in vitro passage or random chemical mutagenesis, with the potential accumulation of unknown and uncharacterized mutations and the risk of reversion. By contrast, recombinant, attenuated vaccines are specifically engineered to inactivate defined target functions with non-reverting mutations.

The rational design behind genetically engineering a recombinant vaccine strain should find the right balance between attenuation and immunogenicity.

One of the most commonly used mutations for generating vaccines for intracellular pathogens such as *Salmonella* and *Shigella* are *aro* gene mutations, which disable the shikimate pathway essential for the biosynthesis of aromatics including the aromatic amino acids [23].

Currently, candidate live vaccine strains are being constructed that combine different mutations, which optimize the balance between attenuation, reactogenicity and immunogenicity, highlighting the need to understand the organisms' physiology and interaction with the host as well as protective immune responses to the disease [24–26].

b) Conjugate Vaccines

The use of protein-conjugated polysaccharides as a tool for the prevention of diseases caused by encapsulated bacteria has proved to be highly successful in the development of effective vaccines against *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae*.

Subunit vaccines based on purified capsular polysaccharide can elicit protective immune responses in adults and are the basis of licensed multivalent pneumococcal and meningococcal polysaccharide vaccines. However, these have not been widely exploited as they were shown to result in short-lived protective immunity and to be ineffective in infants, largely because of their inability to engage a T cell-based immune response. The development of conjugate vaccines derived from polysaccharides of the capsule chemically conjugated to tetanus toxoid or diphtheria CRM197 carrier proteins resulted in a T-dependent antigen capable of protecting young children and providing long-term immunological memory [27].

c) Reverse Vaccinology

Genome mining has revolutionized the approach to vaccine development and provided a new innovation to antigen selection and design. The approach starting from the genomic information leading to the identification of potential vaccine candidates is termed “reverse vaccinology” [28]. The availability of complete bacterial genome sequences offers a comprehensive catalog of genes encoding all the potential protein antigens of a pathogen, with the possibility of rational selection of vaccine candidates rather than empirical testing of antigens, one at a time. Furthermore, the prediction of antigens is independent of the need to culture the pathogen in vitro, to establish the abundance of the antigen, and to understand which antigens will be expressed in vivo. On the basis of the concept that surface-exposed antigens are susceptible to antibody recognition and are therefore the most suitable vaccine candidates, a complete genome can be screened using bioinformatic algorithms to select open reading frames (ORFs) encoding putative surface-exposed or secreted proteins.

Surface proteins are readily identified in genomic data based on the combination of several pieces of information, including the presence of amino acid motifs that are responsible for targeting the mature protein to the outer membrane (signal peptides), to the lipid bilayer (lipoproteins), to the integral membrane (hydrophobic transmembrane domains), or by searching for motifs indicative of surface location such as sortase attachment sites (otherwise known as LPXTG sites) or motifs for recognition and interaction with host proteins or structures (e.g., adhesins having integrin-binding domains).

Furthermore, screening for sequences homologous to those of human proteins for their exclusion in the selection process can help to avoid problems of autoimmunity. Currently available software and bioinformatic techniques for antigen discovery have been recently reviewed [29]. Furthermore, current efforts are aimed at refining algorithms to enable biotechnologists to follow “computer-aided strategies” based on experiments driven by high-confidence predictions. Some groups are currently involved in developing and tuning integrative approaches and user-friendly, automated bioinformatics environments [30]. For example, NERVE (New Enhanced Reverse Vaccinology Environment) represents a user-friendly software environment, which attempts to bring together a similar body of principles into an automated pipeline resembling a reverse vaccinology process.

After candidate surface antigens are identified *in silico*, they are produced *in vitro* and their immunogenicity is assayed to measure their relative power as potential vaccine candidates. The first application of this genomic approach led to the identification of novel vaccine candidates against *N. meningitidis* serogroup B (MenB) [31,32].

B. Vaccine Prices and Costs

Developing country access to vaccines has been critically dependent on differential pricing (also called tiered pricing, equity pricing, and price discrimination), whereby different prices are charged for the same products in different markets.

Under differential pricing, manufacturers charge a much lower price in the poorest developing country markets, allowing these countries access to the product, and charge much higher prices in the industrialized markets, allowing the manufacturer to recoup its research and development expenditures and overheads.

In the 1990s, financing of vaccines for low-income country markets also began changing. The global immunization community formed the GAVI Alliance, and large amounts of funding for the purchase of vaccines for the world's poorest countries were funneled through this public-private partnership.

The costs associated with product development are incurred in four distinct stages: preclinical research, identifying a promising vaccine candidate through early clinical studies in humans, developing and testing a candidate for a target market, and scaling up manufacturing capacity for that market [33–35].

Assuring a vaccine for global use requires two additional stages of investment: relevance, adapting and testing a vaccine to ensure safety and efficacy in additional populations, and supply, ensuring adequate manufacturing capacity and funding/pricing structures to enable broad developing country access. If a new vaccine is to be broadly available at the earliest possible technical and regulatory opportunity, a manufacturer must make development and capital investments explicitly to support supply to the developing world markets.

For there to be a business case for such incremental investments, a return must be available either directly from the developing world market or through other special financing interventions. In the case of vaccines for which no significant industrial market exists, such as

malaria, it follows that the developing country market and associated financing must fund, and provide a return on, the entire investment [35].

Vaccine production has two main stages, bulk production consisting of activities such as growing or producing by fermentation the active ingredients, and purifying the result, and fill/finish consisting of activities such as blending, filling vials, lyophilizing where applicable, labeling, and packaging. All suppliers require production facilities with appropriate quality control and quality assurance that are in compliance with current Good Manufacturing Practice (cGMP), as required by the regulatory authorities. The costs associated with vaccine production can be categorized as variable (unit cost is constant for each vial), semi-variable or batch fixed (costs associated with each batch), and fixed (costs are independent of volume or number of batches and are fixed at the site or company level). Vaccine production is largely a “fixed cost business,” in that the majority of costs are fixed at either the batch or site level (plant, equipment, animals, labor), leaving only a relatively small percentage truly variable (vials, stoppers, raw materials).

In general, the fixed cost nature of vaccine economics means that unit costs fall rapidly with increases in volume. Timing becomes particularly important because the capacity and batch size for a given vaccine are largely determined at scale-up, and are thereafter expensive and time consuming to increase. The cost to produce vaccine varies significantly from product to product (Tremonti G. Advance Market Commitments for New Vaccines: A Tool in the Fight Against Disease and Poverty. Report to the G8 Finance Ministers, London, U.K. Advanced Market Commitments for Vaccines; 2005).

Three types of analyses are commonly used to assess the economic rationale for immunization or other health-related programs: cost-benefit (CB), cost-effectiveness, and cost-utility analyses.

Cost-benefit analyses (CBAs) compare the monetary benefits from implementation of a health program with its monetary costs. All values in a CBA, including health outcomes, are converted to monetary units, resulting in an outcome that is expressed as money saved or spent.

The Cost-effectiveness analysis (CEA) assesses the value of a program by calculating the expenditure per health outcome achieved (net cost/net health effect). Common outcomes include cost per case prevented and cost per death averted.

Cost-utility analysis (CUA) is a subset of CEA. In CUA, as with CEA, outcomes are expressed as cost per change in health status; thus, health outcomes do not need to be converted into monetary equivalents. However, a common metric is established that allows comparison of qualitatively different health outcomes. [36,37] [38].

Cost of Goods Sold, also known as COGS, is one of the most difficult, yet important elements of business to understand. The basic definition of cost of goods sold is: the direct costs attributable to the production of the goods sold by a company. This amount includes the cost of the materials used in creating the good along with the direct labor costs used to produce the good.

One of the main drivers for continuous process improvement is COGS reduction. Manufacturing costs do not in general directly determine vaccine prices, which are set by profit-seeking firms in markets that are more or less competitive. But they do set a floor below which prices cannot fall even in tiered pricing or highly competitive situations. Vaccine production costs include variable costs associated with each dose of vaccine (“cost of goods”), which include the cost of the vaccine components, vials, and so on; “semi-fixed” costs associated with each production batch, including quality tests; and the fixed costs of plant and equipment. Fixed and semi-fixed costs make up the bulk of total cost, typically contributing 60% and 25% respectively; this gives vaccine manufacture large economies of scale, since average costs fall with increasing volume over a large range [39].

The main factors that determine manufacturing cost are vaccine and production technology, presentation, and manufacturing scale. Location of production matters, as some costs are lower in developing countries.

Vaccines of different types can have dramatically different costs of production. At one end of the spectrum, simple live-attenuated vaccines such as oral polio or measles can be produced in very large batches from very inexpensive inputs, and cost no more than a few pennies per dose. At the other extreme, the new pneumococcal conjugate vaccines require the manufacture or purchase of purified bacterial polysaccharides, conjugation of these polysaccharides to a protein carrier, mixing and carefully balancing of component vaccines against as many as 13 bacterial strains or serotypes, and a very large number of quality control steps. These vaccines cost \$1-3 per dose to produce not counting the cost of the plant itself. Production technology

also determines batch size, which is itself an important determinant of cost, since some expenses are incurred on a per-batch basis [40,41].

The cost of producing technologically sophisticated vaccines can be expected to come down over time as firms accumulate experience and as more efficient production technologies are developed. But the nature of some vaccines makes them intrinsically more costly to manufacture than others. For this reason, choices made during the earliest stages of vaccine development have critical consequences for later manufacturing cost. Once a technology has been chosen, producers are largely locked in.

Design choices are also critical, and market incentives in rich countries may drive the development of more complex, and thus expensive, vaccines than are required for public health impact.

Several new vaccines with vital implications for developing countries have come to market recently, every effort must be undertaken to ensure that children in these countries gain access to them. This will depend both on raising additional funds and bringing prices for newer vaccines down by accelerating the entry of emerging suppliers into these markets. Technology and know-how transfer will be crucial, and will also help emerging suppliers to assume a larger role in vaccine research and diagnostic (R&D). And innovative models to stimulate R&D will be critical to meeting still-unaddressed vaccine needs [42].

Vaccine for Developing Countries should be designed to be inexpensive (less than one euro for association like UNICEF and GAVI), stable at room temperature, simple to administer to infants, readily administered by nonprofessionals using a needle-free method and the vaccines formulation would protect against multiple agents [43].

a) The Meningitis Vaccine Project (MVP)

The Meningitis Vaccine Project (MVP) is an intriguing model of vaccine development for developing countries, in which a vaccine with specific characteristics tailored to a particular population is developed at a modest cost and provisions to ensure sustainable access are built in from the start. Although similar to the larger vaccine Product Development Partnership (PDPs), its approach has differed in several ways. The MVP was established in 2001 by PATH and WHO to develop an effective and affordable vaccine to combat the epidemics of bacterial meningitis, caused mostly by group A strains of *Neisseria meningitidis* (“meningococcus”), which plague the so-called “meningitis belt” in sub-Saharan Africa (www.who.int). While polysaccharide vaccines have been available for many years, they have limited duration of protection and are not effective in very young children (WHO. Immunization, Vaccines and Biologicals. www.who.int). So-called “conjugate” vaccines, like the one produced by Sanofi, in theory overcome these disadvantages. But the Sanofi conjugate vaccine, an ACYW-strain tetravalent, is more expensive to produce (four conjugate vaccines instead of just one). Even at a heavily discounted price, it would likely remain far more expensive than a monovalent vaccine.

After identifying and licensing an appropriate conjugation technology from the U.S. FDA (U.S. Food and Drug Administration) and negotiating with several possible industrial partners, the MVP reached agreement with Serum Institute of India (SIIL) to produce the new monovalent meningitis A conjugate vaccine at an affordable price for the African market. In exchange for price and supply commitments, SIIL benefited from transfer of technology and know-how. PATH funded the clinical trials.

Significant progress has been made. In December 2009 market authorization for the Men A conjugate vaccine was granted by the Drugs Controller General of India and the WHO prequalification process has begun. Plans are being made for introduction at public health scale in Burkina Faso in late 2010. The total research and development costs for this vaccine are estimated to be about \$60 million, not including the cost of the manufacturing plant; SIIL has committed about \$15 million to the project.

The MVP’s approach differs in some respects from that of the larger PDPs:

- *Focus on low cost:* Consultations at the start of the project established that to ensure long-term access a vaccine should cost no more than \$0.50/dose. MVP emphasized this requirement in discussions with vaccine manufacturers.
- *Focus on a single candidate and single supplier:* Unlike most product development partnerships that maintain a portfolio of vaccine candidates, MVP focused on a single candidate and on SIIL as the sole supplier. The relatively high probability of success in making a monovalent conjugate vaccine lowered the risk when compared to a malaria or Tuberculosis (TB) vaccine, where the risks of R&D failure are high.
- *Partnership with an emerging supplier:* The choice of an emerging supplier was critical to achieving the goal of low cost, in part because the access to technology and visibility associated with the project made the partnership more attractive to SIIL than to a multinational firm. In addition, producing for a high-volume, low-margin developing country market was consistent with SIIL's established business model.
- *Public sector technology transfer:* The transfer of a non-exclusive license for an efficient conjugation technology through the NIH (U.S. National Institutes of Health) Office of Technology Transfer, coupled with active transfer of know-how, was central to the success of the project.

To a great extent, these choices were made possible because several meningococcal conjugate vaccines had already been developed, the technologies were well understood, and correlates of protection for Meningitis A were known. These data made focusing on one candidate a reasonable bet, allowed emerging suppliers to be considered as manufacturing partners, and kept costs relatively low. The MVP model is therefore particularly suited to the development of adapted versions of vaccines based on established technologies, such as for rotavirus, pneumococcal or HPV vaccines. On the other hand, the development of AIDS, TB and malaria vaccines is far more challenging and probably requires different models.

Other forms of push funding for vaccine development are under discussion, including new funds that would pool donor resources and allocate them among PDPs and possibly firms, proposals to use bond markets to raise long-term funding for PDPs, as well as currency transaction levies or financial transaction taxes.

An alternative to the PDP model of publicly subsidized collaboration with the private sector would be to rebuild capacity in the public sector to develop and test vaccines.

(Special Report: Giving Developing Countries the Best Shot: An Overview of Vaccine Access and R&D, May, 2010)

C. Reaching Every Child: Achieving Equity in Global Immunization

In the year 2000, ambitious millennium development goals were underwritten by all nations. These goals include a two-thirds reduction in child mortality by 2015. For this to be achieved, the full potential of the global EPI network must be exploited to prevent the 1.4 million vaccine-preventable childhood deaths that continue to occur every year. Preventing these deaths requires extending the reach of immunization services to the more than 25% of the world's children who are yet to have regular access to immunization, the most cost-effective of health interventions. Particular attention must be given to increasing coverage among the world's poorest children who are disproportionately affected by vaccine-preventable and infectious diseases. That reaching all children with immunization is a feasible goal is abundantly evident in the progress achieved and experience gained over the past 25 years.

With the increased attention and funding for immunization, mainly through the Global Alliance for Vaccines and Immunization (GAVI) and a realization that immunization, both current and future vaccines, would play a major role in reaching the millennium development goals for child survival, WHO and UNICEF in 2004/2005 developed a strategic document, the Global Immunization Vision and Strategy (GIVS), which outlined the overall vision, direction, and core strategies.

GIVS set new global goals in immunization for routine vaccination, it requires that all countries should reach 90% of their children with vaccines, and in terms of measles control, it dictates that mortality should be reduced by 90% compared to the 2000 level. The document describes four key strategic areas that would allow immunization programs to grow further. The first strategic area “protecting more people in a changing world” outlines strategies to expand the reach of vaccinations both geographically (by targeting the hard-to reach) populations and in terms of age groups, requiring the expansion of vaccination programs to reach children beyond the first year of life. The second strategic area “introducing new

vaccines and technologies’’ describes the strategies needed to support countries to make the decision, and to implement new vaccine or technology introduction. In the third strategic area ‘‘integrating immunization, other linked health interventions, and surveillance in the health systems context,’’ the need for immunization programs to work in coordinated and integrated fashion with other programs, and the basic health system itself is outlined. This area also contains the key strategies required to broaden and strengthen the surveillance and monitoring systems necessary to run a successful public health program.

Finally, in recognition that the immunization program operates in a global context of interdependency, the fourth strategic area ‘‘immunizing in the context of global interdependence’’ describes the necessity of sustainable financing and supply of vaccines of assured quality, as well as communication, information dissemination, partnerships, and global epidemic preparedness.

UNICEF and WHO have substantially enhanced long-term vaccine demand forecasting capacity, improved their knowledge of the vaccine market, increased dialogue with manufacturers, explored arrangements for longer-term funding and contracts for vaccines, and promoted sustainable financing mechanisms.

The challenge will be to exploit the opportunities that exist to establish true equity in global childhood immunization.

3. *SALMONELLA*: a general overview

Salmonella infections in humans are divided into typhoid fever caused by *S. typhi* and *S. paratyphi* and a range of diarrhoeal diseases caused by a large number of non-typhoidal *Salmonella* serovars (NTS). These NTS, which usually have a broad vertebrate host range, show dramatically more severe and invasive presentation in immunocompromized individuals [44].

Typhoid fever (TF) is a more classical systemic infection caused by the typhoid bacillus, *Salmonella enteritica* serovar Typhi (commonly referred to as *S. typhi*), the most common cause of enteric fever, which also includes paratyphoid fever caused by *S. paratyphi* A, B and C. These pathogens only infect humans. The disease is transmitted by ingestion of food, including dairy products, or water contaminated by excreta from patients or chronic carriers or handled by infected persons [45].

A. Bacteriology

The most recent classification, based on DNA sequences, has left only two species, *S. enteritica* and *S. bongori*, further subdivided into subspecies and serovars. To avoid confusion, *S. enteritica* serovar Typhi continues to be referred to as *S. typhi* [46].

According to this system of nomenclature, “*Salmonella typhimurium*” would be renamed “*Salmonella enterica* serotype Typhimurium.” Although this proposal was not formally adopted by the International Committee of Systematic Bacteriology, these names have become accepted for use by the World Health Organization and in publications of the American Society for Microbiology. Most medical laboratories continue to report clinically familiar names, such as *Salmonella typhimurium* or *Salmonella* serotype Typhimurium. Although *Salmonella typhi*, the most frequent causative antigen of enteric fever, may be rapidly identified (serogroup D; Vi-antigen positive) by use of commercial agglutination tests in all laboratories, *Salmonella paratyphi* and the multitude of nontyphoidal salmonellae are, variably, members of serogroups B and D and, less frequently, of serogroups A and C. Final serotyping will often require a reference laboratory and not be immediately available. Fortunately, clinical decision-making is based primarily on the host, severity of illness, and antimicrobial

susceptibilities, and not specific serotype. Identification of *Salmonella* species in the bacteriology laboratory is not difficult. The best recovery of such species from fecal samples can be achieved by the use of direct plating and inoculation of standard enrichment broths [47,48].

Upon ingestion, typhoid bacilli rapidly penetrate the small intestinal mucosa by transcytosis through M cells and enterocytes, and are taken up by macrophage or diffuse into mesenteric lymph nodes. A primary bacteremia follows and the pathogen rapidly attains intracellular haven throughout the reticuloendothelial system. This is followed by a sustained secondary bacteremia associated with clinical illness. *S. typhi* also shows remarkable predilection for the gall-bladder where infection tends to become chronic, especially in individuals with a pathologic gall-bladder condition [49] .

B. Disease Burden

a) TYPHOID FEVER

Typhoid Fever (TF) is spread by the fecal-oral route and closely associated with poor hygiene, lack of clean drinking water and inadequate sanitation. The disease is almost exclusively transmitted by food and water contaminated by the faeces and urine of patients and carriers. Polluted water is the most common source of typhoid transmission. In addition, shellfish taken from sewage-contaminated beds, vegetables fertilized with night-soil and eaten raw, contaminated milk and milk products have been shown to be a source of infection. Although TF has practically disappeared from industrialized countries, it remains a serious public health problem in several Asian regions of the former USSR and in parts of South and South-East Asia, Africa and South America. In the last outbreak in the Democratic Republic of Congo, between 27 September 2004 and early January 2005, no less than 42 564 cases of typhoid fever were reported, including 214 deaths and 696 cases of peritonitis and intestinal perforations. Also, multiresistant strains of *S typhi* are becoming increasingly common worldwide, further compounding the risk to people living in regions with high endemic disease and to travelers [50,51] [52].

Strains resistant to chloramphenicol and other recommended antibiotics (ampicillin, cotrimoxazole and even ciprofloxacin) have become prevalent in several areas of the world [53].

TF is characterized by the sudden onset of sustained fever, severe headache, nausea, abdominal pains, and loss of appetite, constipation or sometimes diarrhea. The illness can last for several weeks and even months. The most frequent complications, which arise with a frequency of 1% to 4%, include gastro-intestinal bleeding and intestinal perforation [54]. Severe neurological forms also have been described with mental dullness, stupor, delirium and shock. Hospitalization of TF cases varies from 10% to 40% of cases and usually lasts for 10-15 days or more. Case-fatality rates, which varied from 10% to 30% before the advent of antibiotics, has now been reduced to about 1%-4% with appropriate antibiotic therapy [54]. Paratyphoid fever, which is caused by any of three serotypes of *S. paratyphi* A, B and C, is similar in its symptoms to typhoid fever, but tends to be milder, with a lower fatality rate [55]. People can transmit TF as long as the bacteria remain in their body; most people are infectious prior to and during the first week of convalescence, but 10% of untreated patients will discharge bacteria for up to 3 months. In addition, 2–5% of untreated patients will become permanent, lifelong carriers of the bacteria in their gall-bladder.

The true burden of TF in developing countries is difficult to estimate. According to recent estimates, 22 million (range 16 million - 33 million) cases occur each year causing 216,000 deaths, predominantly in school-age children and young adults. Asia, with 274 cases per 100,000 persons has the highest incidence of TF cases worldwide, especially in Southeast Asian countries and on the Indian subcontinent, followed by sub-Saharan Africa and Latin America with 50 cases per 100,000 persons. In an urban slum in Dhaka, incidence of bacteremia TF was found to be 390/100,000 population, with a 9-fold higher risk for pre-school children than for older persons [56].

Recent prospective population-based disease-surveillance studies supported by the Bill and Melinda Gates Foundation and conducted by the Diseases of the Most Impoverished (DOMI) Program at five sites in China, India, Indonesia, Pakistan and Vietnam revealed high rates of TF among children in urban slums, including children below 5 years of age. In three urban slums in Karachi, Kolkata and North Jakarta, incidence of blood-confirmed TF cases among children 5 to 15 years of age ranged from 180 cases to 494 cases per 100,000 [57,58].

The introduction of TF vaccines in routine vaccination programs in Asia would be highly beneficial in view of the burden of disease and cost of illness to governments and individuals [59]. But, so far, only two countries, China and Vietnam, have incorporated typhoid vaccination into their routine immunization programs, and only in a limited fashion. The Delhi State, India, also introduced vaccination in 2004 in 2-5 years old children through community-based campaigns. The reason why these efforts have not been more generalized lies in part in the fact that most developing countries are uncertain of their true TF disease burden, due to lack of rapid diagnostic tools, infrequency of laboratory testing and poor reporting system (Report of the Meeting on Typhoid Fever, a Neglected Disease: Towards a Vaccine Introduction Policy). Most cases of TF in industrialized countries are imported cases from endemic countries [60].

b) NON-TYPHOID SALMONELLAE

Non-typhoid salmonellae are among the primary causes of food-borne gastroenteritis. Most infections result in self-limiting diarrhea and do not require antimicrobial treatment. However, invasion of the bloodstream and life-threatening metastatic infections, such as meningitis, are more common in infants, the elderly and immune-compromised patients [61].

Annually, approximately 40 000 cases of non-typhoid salmonellosis are reported to the Centers for Disease Control and Prevention (CDC). These numbers underestimate the magnitude of the problem, as many cases of salmonellosis are not reported because the ill person either does not visit a physician, no specimen is obtained for laboratory tests or the laboratory findings are not communicated to the CDC. Taking into account the degree of under-reporting, the CDC estimates the annual number of non-typhoidal salmonellosis cases in the US to be approximately 1.4 million [62,63]. Furthermore, with an estimated 582 annual deaths resulting from non-typhoid salmonellosis this pathogen is the leading cause of foodborne infections with lethal outcome in the US. The most common vehicles of transmission are meat, meat products, eggs and egg products that contain *Salmonella* serovars because animals are infected or because fecal contamination occurs during processing [64]. The majority of human cases are caused by only a few non-typhoid serovars. For instance, approximately 60% of human cases reported to the CDC in 1995 were caused by four serovars,

including *Salmonella enteritidis* (24.7%), *S. enterica* serovar Typhimurium (23.5%), *S. newport* (6.2%) and *S. heidelberg* (5.1%) [CDC, *Salmonella* surveillance: annual tabulation summary 1993–1995 US Department of Health and Human Services, CDC, Atlanta, 1996]. These four serovars also represented 46.4% of strains isolated from non-human sources that year. The dominance of only a few serovars is even more pronounced in Germany, where *S. enteritidis* (61.3%) and *S. Typhimurium* (23.4%) accounted for more than 80% of human isolates reported to the enteric reference center at the Robert Koch Institute in 1995.

The epidemiological surveys by the Central Veterinary Laboratory revealed a steady increase in multi-drug resistant *S. Typhimurium* isolates in England and Wales. The percentage of *S. Typhimurium* isolates resistant to multiple antibiotics increased from 5% in 1981 to 19% in 1990 [65]. Multiple resistance has continued to increase, resulting in 81% of *S. Typhimurium* isolates from England and Wales being multi-resistant in 1996 [65].

However, antimicrobial resistance, sometimes more prevalent than in *S. typhi*, is clinically relevant because 3–10% of these infections can progress to life-threatening bacteraemia, particularly in young children and HIV-infected people [66]. In Indonesia, Tjaniadi although *S. typhi* and *S. paratyphi* isolates recovered between 1995 and 2001 were universally susceptible to commonly used antimicrobials, *Salmonella enteritidis* isolates were resistant to most of the antimicrobials tested, with the exception of the fluoroquinolones [67]. Similarly, Zimbabwean study reported much lower rates of resistance among *S. enteritidis* [68,69], more than 50% of non-typhoidal salmonella isolates from children in Kilifi, Kenya, were multidrug resistant [69,70]. The potential consequences of these pockets of multidrug-resistant salmonella could be increased health costs even if infection rates are not increased. In a controlled study, Martin and co-workers found that patients infected with multidrug-resistant *S. enterica* serovar Typhimurium were more likely to be hospitalised than those infected with susceptible strains [70].

Available data suggest that resistance has reached unacceptable levels in the pathogens most common in developing countries and that trends show further increases. Resistance appears to have emerged and spread rapidly in many areas, with important consequences for individual patients and public health. The agents most affected are inexpensive, older antimicrobials, which in many cases are all that are available or affordable. Widespread selective pressure and

efficient dissemination channels for multidrug-resistant organisms are major factors that may have contributed to the rapid emergence and spread of resistant organisms.

A pressing and unmet need exists to alter these factors in areas where susceptibility has been recorded and to identify other contributors to these alarming trends.

National and international policy decisions backed by political and social will are necessary to provide a more accurate assessment of the problem and interrupt the unacceptable trends [71].

C. Clinical Spectrum

Salmonella infection may appear to be one of several distinct clinical syndromes, or symptoms may overlap in its clinical presentation. The major disease states produced by salmonellosis are enterocolitis (gastroenteritis), enteric fever, bacteremia, focal infection and chronic carrier state [46].

a) Enterocolitis

Localized acute intestinal infection is the most common form of salmonellosis. In the small intestine, epithelial invasion occurs but without extensive destruction of intestinal mucosa [72].

The organisms reach the lamina propria where they multiply and elicit an inflammatory response. In

S. typhi, this response may trigger production of predominantly mononuclear cells, whereas non-typhi

Salmonella infection generally evokes polymorphonuclear neutrophilic reaction. It is also now known that colonic involvement occurs with acute salmonellosis (hence the term "enterocolitis"); histological changes include edema, inflammation, erosion and microabscesses [72,73].

Non-typhoid Salmonella serotypes, particularly *S. Typhimurium*, are most commonly responsible for acute enterocolitis. Although *S. typhi* may produce enterocolitis and cause diarrhea, constipation may be an equally or a more common gastrointestinal symptom with this strain of Salmonella.

Clinical expression of acute enterocolitis may occur as soon as 8 hours or as late as 72 hours following ingestion of *Salmonella* organisms. Symptoms may begin with transient nausea and vomiting followed by myalgia, headache, fevers, chills, diarrhea and abdominal cramps [74]. Generally, the stools are not bloody, are of moderate volume and loose in character. Occasionally, the disease may be severe, associated with fluid and electrolyte depletion leading to hypovolemic shock, blood loss, and toxic dilatation of the colon or bacteremia, particularly in those patients with increased susceptibility to salmonellosis. Symptoms of fever last less than 48 hours and diarrhea usually abates within one week, although patients with colonic involvement, intra-abdominal suppuration or other complications may have a prolonged course of fever, loose stools and abdominal complaints. Management will depend on severity of illness and any associated complications [75].

b) Enteric Fever

Enteric fever is clinically caused by *S. typhi* and hence called typhoid fever. A clinical syndrome nearly identical to typhoid fever but caused by *S. paratyphi* A, B or C was previously designated paratyphoid fever. However, because other serotypes may also cause enteric fever, it is more conventional now to ascribe paratyphoid fever to any serotype other than *S. typhi* causing this syndrome [76].

Following ingestion of the organism, an incubation period of approximately ten days may be followed by bacteremia and the onset of the clinical features of enteric fever. Fever, chills, headache and gastrointestinal symptoms are the most important presenting complaints of patients with enteric fever. Interestingly, cough and bronchitic symptoms occur in more than half of the patients, but documented pneumonia is uncommon. A variety of other nonspecific complaints such as myalgia, arthralgia, weakness or sore throat may also occur. Physical findings are primarily limited to the abdomen [76].

Although paratyphoid fever is nearly identical to typhoid fever in its clinical features, relative bradycardia has been reported in 50 % to 100 % of cases. Neuropsychiatric manifestations of enteric fever are nonspecific but may occur in up to 75 % of patients [77,78]. Confusion, stupor and delirium are the most common findings, occurring in more than 50 % of cases.

Other diffuse and focal neurological deficits and gross schizophrenic psychoses have also been reported [77,78].

Complications of enteric fever, other than death, can be classified as abdominal or extra-abdominal.

Abdominal complications that are most serious are intestinal perforation (1-5 %) and intestinal hemorrhage (1-21 %). Complications occurring outside the abdomen include the neuropsychiatric features, localized infections (meningitis, endocarditis, arthritis, osteomyelitis, pneumonia), bone marrow suppression, parotitis, decubitus ulcer, renal failure and chronic carrier state.

The antimicrobial chemotherapy that has been used to treat enteric fever has been primarily chloramphenicol, ampicillin or trimethoprim-sulfamethoxazole [79].

Resistance to ampicillin may also occur, but less often than to chloramphenicol. Trimethoprim-sulfamethoxazole resistance of *S. typhi* and other *Salmonella* species is uncommon even though resistance to sulfonamides alone may be present [79].

c) Chronic Carrier State

The chronic carrier state is defined as the excretion of *Salmonella* for more than one year.

Chronic enteric carriers who excrete organisms in the feces are the most common, although occasionally chronic urinary carriers are found. Chronic carriers are asymptomatic. The chronic carrier state occurs more frequently following *S. typhi* infection; the incidence is 2-4%.

Chronic carriers following non-typhoid salmonellosis is uncommon [80].

The chronic enteric carrier state of *Salmonella* increases with antigen and is associated with biliary tract disease. A chronic urinary carrier state is more commonly associated with schistosomiasis of the urinary tract. Management should be directed first toward determining the presence or absence of biliary tract disease. The antimicrobial drug of choice is ampicillin (not chloramphenicol, which has a poor success rate) [81].

D. Vaccines already available on the market

a) *The Vi polysaccharide vaccine*

The A subunit of *S. typhi* polysaccharide was developed as a vaccine in the 1980s in the laboratory of John Robbins at the NIH and licensed to Sanofi-Pasteur. The vaccine is based on purified Vi antigen, a linear homopolymer of galacturonic acid that is purified from the bacteria. It is produced by several manufacturers including developing country manufacturers. The vaccine is progressively introduced into school attending children vaccination programs in Asian countries [82] [83,84].

Its efficacy was demonstrated in multiple randomized trials. One of the first trials in Nepal involved persons antigened 5-44 years and showed a 75% protection antigenainst TF during 20 months of active surveillance. Another study in South Africa demonstrated a 64% protective efficacy after 21 months in children antigened 5-16 years, declining to 55% at 3 years after vaccination [85] [86]. A locally produced Vi vaccine prepared by the Shanghai Institute of Biological Products, which was evaluated in a randomized placebo-controlled, double-blind trial on about 60 000 5-19 years old children in China showed a 69% efficacy antigenainst blood culture-confirmed TF over 19 months [87]. The Vi vaccine is considered to be protective for at least three years; it also has demonstrated a remarkable safety profile. Several high-quality producers from developing countries have acquired the technology to produce it at low cost, including two producers in China.

Vi is poorly immunogenic in infants and cannot be used to vaccinate children less than 2 years of antigen. This has prompted the development of a Vi conjugate vaccine at the National Institutes of Health, USA, using recombinant *Pseudomonas aeruginosa* exotoxin A as the protein carrier (Vi-rEPA).

b) *The live attenuated Ty21a vaccine*

The attenuated *S. typhi* strain Ty21a was generated in Switzerland by chemical mutagenesis of wild-type strain Ty2 and developed as the first live oral typhoid fever vaccine. The strain is characterized as lacking both a functional galactose-epimerase (galE) gene and the Vi antigen,

although other mutations in the genome probably are responsible for the attenuated phenotype. The vaccine was extensively tested in Alexandria, Egypt, where the liquid formulation showed 96% protection for 3 years, and in Santiantigeno, Chile, where it was found to elicit a 67% protection against blood culture-confirmed TF over 3 years and 62% over 7 years[88]. The liquid formulation was shown to be 78% protective at 5 years. Ty21a is therefore considered to provide protection for at least 5-7 years. The vaccine was less efficacious when tested in Indonesia, where protective efficacy was found to be only 33%-53%, suggesting that in-the-field effectiveness in TF hyper-endemic areas, such as Indonesia, were quite lower than in areas with lower incidence of the disease, such as Egypt or Chile. In Chile, Ty21a also showed 42%-56% cross-protection against paratyphoid fever caused by serovar *Paratyphi* B [89].

c) Other live attenuated S. typhi vaccines

Several live attenuated *S. typhi* strains are being developed to be used as oral TF vaccines. In preliminary clinical trials, these strains seem to be even more immunogenic than Ty21a [90]. Perhaps the most advanced of these live attenuated candidate vaccines is Ty800, a *phoP/phoQ* deletion mutant of Ty2, which is developed by AVANT Immunotherapeutics [91] and has passed Phase II trials. The vaccine has been shown to stimulate vigorous IgA and serum O-antibody responses in volunteers.

Another attenuated strain is CVD909, an *aroC/aroD/htrA* deletion mutant which was engineered to constitutively express the *S. typhi* Vi antigen. The strain, which induces anti-Vi antibodies in orally vaccinated subjects [92].

The third live attenuated vaccine candidate is ZH09, a *Salmonella* Typhimurium strain deleted of the *Salmonella* pathogenicity island [93].

A killed but metabolically active *Salmonella* Typhimurium strain, CKS362, is also being developed as a candidate *Salmonella* vaccine[94].

In addition, an attenuated *S. typhi* Ty2 strain with deletions in *ssaV* and *aroC* genes has been developed as a live vector (*spi*-VEC) for oral vaccines antigenainst TF and ETEC diarrhoea [95].

d) Salmonella Native Outer Membrane Vesicles

Salmonella spp produces Native Outer Membrane Vesicles (NOMV) containing OmpA and innate TLR ligands. Antigens in NOMV are protected from protease digestion and also recognized by antigen-specific T cells [96].

NOMV contain TLR ligands and *Salmonella* T cell antigens, important findings that link NOMV properties with innate and adaptive host defense mechanisms. Little is known about the interactions of host-immune cells with NOMV, which may influence the development of *Salmonella*-specific immunity during infection *in vivo* [97] although relatively few studies have focused on NOMV produced by salmonellae [97].

Salmonella NOMV are proinflammatory and immunogenic facsimiles of the parental bacteria, and therefore could stimulate development of both innate and adaptive immunity as the ability of NOMV to stimulate responses for the activation of T cells, such as DC maturation (MHC-II, CD86) and proinflammatory cytokine production (TNF-, IL-12) [98].

Salmonella NOMV possess important proinflammatory and antigenic properties similar to the parent bacteria and it is demonstrated that NOMV have the intrinsic combination of antigens and adjuvant properties required of an effective non-replicating complex vaccine to stimulate *Salmonella* immunity.

NOMV could be an additional means to induce pathogen-specific immunity, and are therefore attractive vaccine candidates [99].

4. CELL ENVELOPE OF GRAM-NEGATIVE BACTERIA

The hallmark of Gram-negative bacteria is their cell envelope, which is composed of two membranes (the inner and outer membranes) separated by a compartment (the periplasm) that contains a thin peptidoglycan (or murein) layer. Although both the inner and outer membranes are lipid bilayers that contain proteins, there are dramatic differences in their structure and composition, reflecting their different functions and the dissimilar environments that they contact [100].

The inner membrane (IM) is in direct contact with the cytoplasm and the periplasm, whereas the outer membrane (OM) separates the periplasm from the external environment. Because of its physical properties, the OM functions as a selective barrier that prevents the entry of many toxic molecules into the cell, a property that is crucial for bacterial survival in many environments [101].

All Gram-negative bacteria have an OM, and although there is some variation among different species, the overall structure and composition are largely conserved [101,102].

A. The inner membrane

The IM is a bilayer composed of phospholipids and proteins. Although the phospholipid content varies between bacteria, in *E. coli* 70–80% is phosphatidylethanolamine, 15–20% phosphatidylglycerol and 5% or less cardiolipin [103]. IM proteins can be of two types, integral IM proteins and lipoproteins. Integral IM proteins span the IM with α -helical transmembrane domains, and lipoproteins are anchored to the outer leaflet of the IM by lipid modifications of the N-terminal cysteine residue of their mature form [104]. Many IM proteins function in small-molecule transport, but they also carry out various other cellular functions. All of the membrane-bound biochemical processes that occur in intracellular organelles in eukaryotic cells, such as oxidative phosphorylation, lipid biosynthesis and protein translocation, occur in the IM in bacteria.

B. The periplasm

The periplasm comprises ~10% of the cell volume and is a highly viscous compartment that is occupied by soluble proteins and the peptidoglycan layer. The periplasm is an oxidizing environment, and it contains enzymes that catalyse the formation of di sulphide bonds [105].

Periplasmic proteins participate in small-molecule transport or the breakdown of polymers to ‘bite-size’ compounds [105]. However, many molecular events that are crucial for cell survival also occur in the periplasm. Protein folding and trafficking factors that build and maintain the cell envelope, even in times of stress, are located here [106]. Furthermore, the sensing domains of most IM receptor proteins, such as the histidine kinases of most two-component systems, read environmental cues from this location. Remarkably, as the periplasm is devoid of ATP, all of this protein folding, trafficking and degradation occurs in the absence of an obvious energy source [107].

The peptidoglycan layer is located in the periplasm and serves as an extracytoplasmic cytoskeleton that contributes to cell shape and prevents cells from lysing in dilute environments. It is composed of glycan chains that are crosslinked by oligopeptides, but there are also proteins associated with the peptidoglycan layer as the Lpp (Braun’s lipoprotein) covalently anchors the peptidoglycan layer to the OM [108].

C. The outer membrane

The OM is an unusual membrane in terms of its structure and composition. Unlike most other prokaryotic and eukaryotic membranes, it is highly asymmetric, with the inner leaflet composed of phospholipids and the outer leaflet composed mainly of LPS [109]. The overall phospholipid composition is slightly different from that of the IM, as the OM is enriched in saturated fatty acids and phosphatidyl ethanolamine [110,111].

LPS is composed of lipid A, a core oligo saccharide and an O-antigen polysaccharide of various lengths [112]. Many laboratory strains of *E. coli* lack O-antigen and mutants in the core ‘deep-rough’ mutants can be obtained. However, lipid A and the inner-core sugar KDO (3-deoxy-d-manno-oct-2-ulosonic acid) are essential for growth of *E. coli*.

LPS is essential for the barrier function of the OM, as was shown decades ago by the increased OM permeability of ‘deep rough’ mutants [113]. The strong lateral interactions between LPS molecules allow LPS to be highly compacted, giving the OM an almost gel-like appearance that is crucial for its barrier function. These interactions include interactions between fatty-acid chains and between the sugar components [114].

In addition to lipids, there are two types of proteins in the OM, lipoproteins and integral Outer Membrane Proteins (OMPs). About 90% of all lipoproteins are located in the OM, where they are anchored to the inner leaflet in the same fashion as the lipoproteins anchored to the outer leaflet of the IM [104,114]. In fact, the N-terminal lipid modifications that are present in all membrane lipoproteins are made at the periplasmic face of the IM. By contrast, integral OMPs are different from integral IM proteins. Instead of having α -helical transmembrane domains, OMPs span the OM with amphipathic antiparallel β -strands that adopt a barrel like conformation which allows many of them to serve as channels [115]. As membranes are fairly impermeable to hydrophilic solutes, the channels that OMPs form are vital for the intake of nutrients and the excretion of toxic waste products. OMPs that do not function as channels can serve as enzymes as well as adhesins [115].

Furthermore, the OM contains other lipopolysaccharides, such as enterobacterial common antigen (ECA), and, under certain conditions, a polysaccharide capsule can be produced as an additional protective extracellular layer [116]. The OM also serves as the anchor for surface organelles such as pili that have a crucial role in pathogenesis [117]

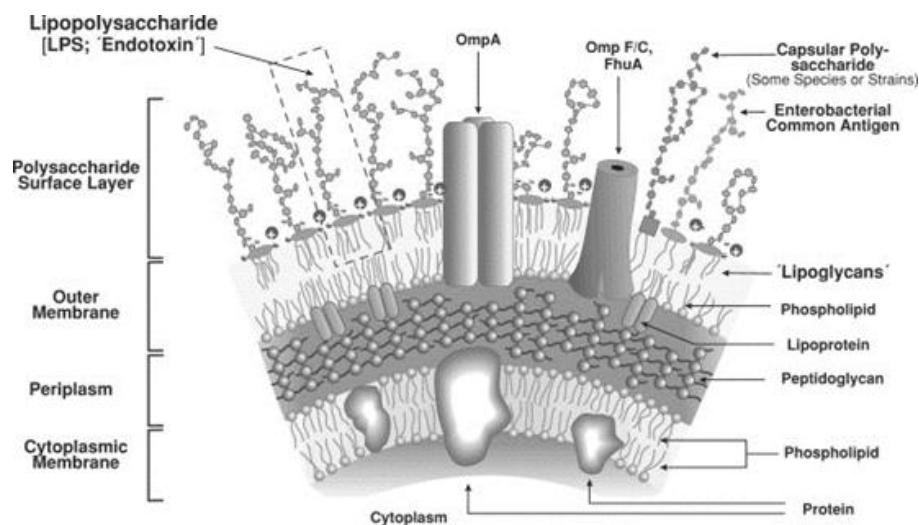


Figure 1. Cell envelope of gram-negative bacteria

D. Lipopolysaccharide (LPS)

LPS is a complex glycolipid exclusively present in the outer leaflet of the OM of gram negative bacteria. It consists of a hydrophobic membrane anchor, lipid A, substituted with an oligosaccharide core region, which in some bacteria (e.g., in most *E. coli* strains, but not in the laboratory strain *E. coli* K-12 and also not in *N. meningitidis*) is extended with a repeating oligosaccharide, the O-antigen. These different LPS constituents are synthesized at the cytoplasmic leaflet of the IM. The lipid A moiety of LPS is rather well conserved among gram-negative bacteria. It usually consists of a β -1,6-linked d-glucosamine disaccharide carrying ester- and amide-linked 3-hydroxy fatty acids at the 2, 3, 2', and 3' positions and phosphate groups at the 1 and 4' positions. The primary 3-hydroxy fatty acids may be substituted with secondary fatty acids. The lipid A biosynthetic pathway, also known as the Raetz Pathway [118], has been characterized in detail, mostly in *E. coli* and *Salmonella* Typhimurium, but it appears to be highly conserved among other gram-negative bacteria [118]. Remarkably, although LPS has so far only been detected in gram-negative bacteria, homologs of the genes encoding the lipid A biosynthetic enzymes have also been found in sequenced plant genomes [119].

The core oligosaccharide is much more variable between bacterial species. In addition, a huge amount of LPS heterogeneity is created by numerous modifications of both the lipid A part and the core part of LPS [120]. The modifying enzymes, which are localized in different cellular compartments, are not generally conserved thus different species can express uniquely modified types of LPS [121]. The O-antigen, if present, is the most variable part of LPS and shows even a high degree of variability between different strains of the same species [122].

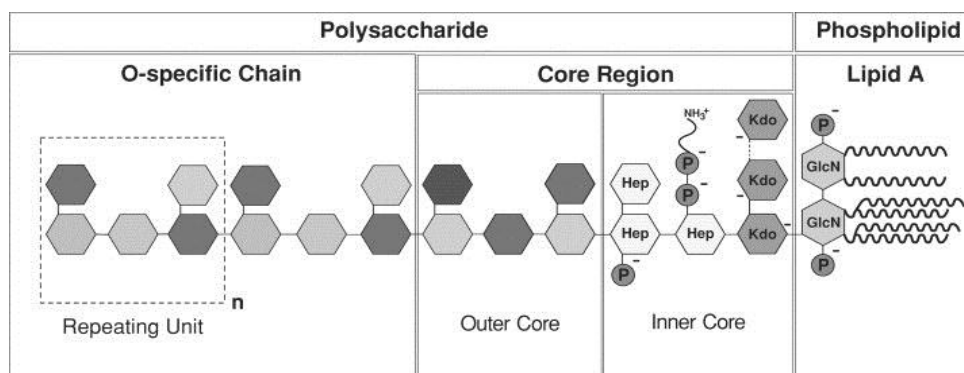


Figure 2. Structure of Lipopolysaccharide

E. O-Antigen

O-antigen, which is the most variable part of LPS, is composed of many repeats of O units. The variation of O-antigen lies in the sugar composition, the arrangement of the sugars, and the linkages between the sugars within the O unit as well as the linkages between O units [123]. O-antigen is an important component in the resistance to serum-mediated killing, phagocytosis, and killing by cationic peptides. O-antigen is synthesized separately before ligation to lipid A-core to form LPS. The O unit is synthesized by sequential transfer of sugars from respective sugar nucleotides to the carrier lipid, undecaprenol-phosphate (UndP) at the cytoplasmic side of the inner membrane. The O units are then translocated to the periplasmic side of the membrane by the O unit flippase Wzx and polymerized by Wzy to form a long chain O antigen before ligation to a preformed lipid A-core molecule [124].

O-antigen is highly immunogenic after immunization with whole cells or with LPS, the O-antigen immunogenicity may depend on the adjuvant effect of the lipid-A. O-antigen, as it is a polysaccharide, elicits only T-cell-independent, that is, essentially antibody, responses. The antibody-antigen complex then activates the complement system, leading to the production of the MAC or to phagocytosis [125,126].

Some O-antigens resemble host components and probably facilitate invasion by mimicry of the host.

Other O-antigens lead to specific interactions and perhaps act as adhesins. Most O-antigen forms occur in several subspecies, indicating that there has been extensive transfer of genes involved in O-antigen biosynthesis, presumably due to selection of one O-antigen form over another [126].

Many bacterial species have extensive variation in O-antigen structure and this polymorphism must be maintained by some form of balanced selection, such as niche-specific selection, with each clone having an O-antigen that is appropriate to its niche [127] .

5. GMMA: GENERALIZED MODULES FOR MEMBRANE ANTIGENS

In NVGH to distinguish the naturally shed blebs to the detergent-derived vesicles [128] we used the term GMMA (Generalized Modules for Membrane Antigens), indicating blebs released from the surface of living Gram-negative bacteria [123,129].

By electron microscopy, GMMA appear spherical with a bilayer membrane, electron-dense luminal content, and an average diameter of 50–250 nm, depending on the strain [130]. GMMA are pelletable from a cell-free supernatant and, due to their lipid content, GMMA will fractionate into lighter density fractions than soluble secreted proteins [131].

Biochemical analysis of density gradient-purified GMMA demonstrated that native GMMA consist only of the protein and lipids of the OM and periplasm and do not contain IM and cytoplasmic components [132]. Enrichment and exclusion of GMMA membrane and soluble cargo as compared with their concentrations in whole bacteria point to GMMA as a specific secretory mechanism. It is quite difficult to assess accurately the enrichment or exclusion of cargo. The localization of a protein in the whole cell influences its availability as GMMA cargo. For instance, a periplasmic protein that has affinity to the inner leaflet of the OM is more likely to appear “enriched” in GMMA when compared with the soluble periplasmic fraction of the whole cell, yet it may not be enriched when considering its concentration in the whole cell. Unfortunately, these affinities are often not known. Thus accurate enrichment/exclusion values are best determined by comparing the quantities of a component in GMMA and in whole cells relative to the quantities of other components [133].

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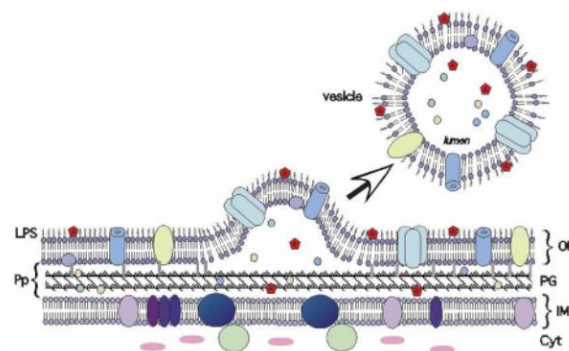


Figure 3. GMMA release from gram negative bacteria

Vesiculation is an ubiquitous process for Gram-negative bacteria grown in a variety of environments including liquid culture, solid culture, and in biofilms [130]. In general, 0.2%–0.5% of OM and periplasmic proteins are packaged in GMMA from *E. coli* and demonstrating that, OM GMMA formation and release is an energy sink [134]. In light of this, bacteria are unlikely to produce GMMA without function. In nonpathogenic bacteria, GMMA sometimes play a protective role.

Although OM GMMA production has been observed for more than 50 years, the machinery that allows

GMMA secretion while maintaining bacterial viability remains elusive. Many theories exist on the mechanism of vesiculation based on biochemical and genetic data and are reviewed elsewhere [135]. Based on budding events viewed by electron microscopy, outer membrane GMMA are thought to form when the OM bulges and pinches off, encapsulating soluble periplasmic cargo [136]. The maximum rate of GMMA production occurs during the end of log phase growth and, as documented for *E.coli*, *Vibrio cholerae*, and *B. melitensis*, and GMMA are abundant at sites of cell division [137].

GMMA could be used as nutrient sensors and transporters, and their presence has been postulated to represent an alternative mechanism for survival in nutrient-limited systems [138]. Moreover, the release of GMMA increases when bacteria are exposed to conditions such as high temperature, exposure to antibiotics or serum, or nutrient deprivation [139].

GMMA from both intracellular and extracellular bacterial pathogens have been identified in diverse host tissues, revealing the ability of GMMA to access a variety of environments within the host.

GMMA have also been detected in the fluids from infected hosts, demonstrating their ability to disseminate distant from the site of infection. The intracellular pathogen *Salmonella* Typhimurium secretes GMMA when growing intracellularly as well as into the supernatant of bacterial cultures [140].

LPS serotype switching, oxygen stress, the availability of iron, and the presence of antibiotics are all pertinent to pathogenic bacteria living inside the host, and these conditions impact GMMA production, toxicity, and composition. Altering expression of LPS O-antigens is a well characterized defense strategy for pathogens to evade the host response [141] .

The presence and type of LPS O-antigen as well as the indirect effect of oxygen stress on the LPS structure may influence the physical ability of the membrane to bulge and initiate the formation of GMMA [142].

A. Biogenesis of gram negative bacterial GMMA

The mechanisms by which Gram-negative bacteria shed GMMA have not been fully determined, however some experiments suggest three models [143] :

1. GMMA are generated by the loss of cell envelope integrity that occurs when the outer membrane expand more quickly than the underlying peptidoglycan layer
2. Formation of GMMA is linked to the turgor pressure of the cell envelope, which changes with the accumulation of peptidoglycan in the periplasm
3. Some signal molecules enhance the anionic repulsion between LPS by destabilizing the Mg^{2+} and Ca^{2+} salt bridges in the outer membrane, thereby causing membrane blebbing

These three mechanisms may not mutually exclusive and may contribute collectively to the biogenesis of bacterial GMMA [143].

However, the release of GMMA increases when bacteria are exposed to conditions such as high temperature, antibiotics or nutrient deprivation [138] .

Alteration of the Tol-Pal system [123,129,144,145] increase the production of GMMA and it is a very attractive for rapidly recovering large amounts of GMMA from gram negative bacteria such as *E.coli*, *Shigella* spp and *Salmonella* spp [146,147]

In some study is show that the peripalsmic production of soluble protein domains interact with the Tol-Pal system, increasing the production of GMMA and it is very attractive for rapidly recovering large amounts of GMMA [147].

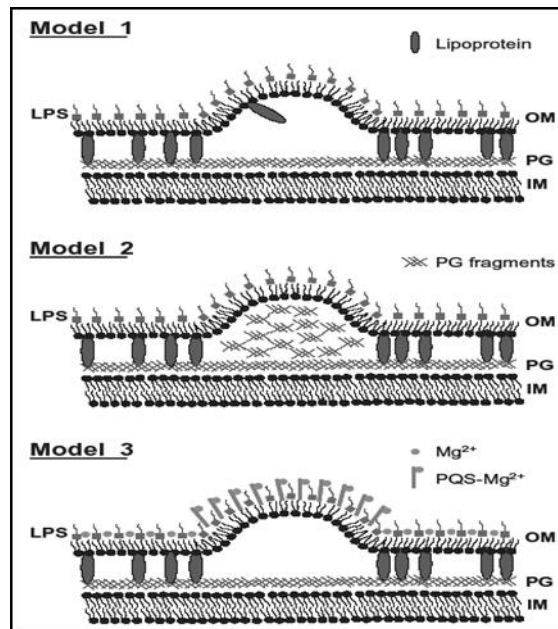


Figure 4- Proposed models for biogenesis of Gram-negative bacterial GMMA.

Model 1: GMMA originate from regions on the cell surface where peptidoglycan-associated lipoproteins are missing due to the faster expansion of the outer membrane than the underlying peptidoglycan layer.

Model 2: accumulation of peptidoglycan fragments in the periplasm causes increased turgor pressure, thereby increasing blebbing of the outer membrane.

Model 3: ionic interactions between quinolone (PQS) and Mg²⁺

B. Inter-bacterial activities

Some bacteria use GMMA as an offensive tactic to gain a growth advantage over other bacteria. Protease and toxin-containing GMMA from *E. coli*, *Shigella*, *Actinobacillus*, *Pseudomonas*, and *Borrelia*, can interact with bacteria possibly via a fusion or adherence mechanism. GMMA proteins that can adhere to the external surface of the bacteria are associated with the external surface of GMMA. Proteins and lipids of the IM and cytosolic content are excluded from GMMA. GMMA are likely to bud at sites where the links between the peptidoglycan and OM are infrequent, absent, or broken [148,149].

The composition of GMMA from pathogens should reflect the state of the bacterial OM, and therefore depend on the site of culturing of the bacteria. Toxins associated with the external GMMA surface act as adhesins, for some GMMA, and the interaction between the toxin and the eukaryotic cell receptor governs subsequent uptake of the GMMA into the host cell. Binding of GMMA to host cells can also influence binding of bacteria to those cells. This binding competes for bacterial cell receptors and may be due to outer membrane. Rather than inhibiting bacterial binding, GMMA enhance the adherence of the pathogen to the epithelial cells.

GMMA have been proposed to act as long-range virulence factors that can protect luminal cargo from extracellular host proteases and penetrate into tissues more readily than the larger bacteria [150].

C. Toxin transporters

Active toxins are associated with several pathogen-derived GMMA and some of the toxins appear to be enriched in GMMA. In some cases, GMMA associated toxins are more active than the toxins alone, and some toxins are associated with the exterior surface of GMMA. The host cell response to the delivery of GMMA-associated toxin is likely to be quite different from that to the delivery of soluble toxin if the uptake of GMMA-associated toxin is coincident with the uptake with GMMA [151].

In addition to specific virulence factors, GMMA contain compounds that are recognized by eukaryotic cells in the innate and acquired immune response pathways. Considering that properties of the OM change during a bacterial infection (such as the O-antigen of LPS), the

impact of GMMA on the modulation of the immune system will also vary during the course of infection.

This is not surprising since GMMA contain a natural adjuvant (endotoxin) and surface-localized bacterial antigens. OMPs present in GMMA are all biologically active molecules that can activate immune cells via Toll-like receptors (TLRs) and induce leucocyte migration [152,153].

6. NVGH PLATFORMS

NVGH and NV&D are investigating the use of GMMA as new vaccines. NVGH has developed new methods to generate hyper-blebbing strains and to collect GMMA from bacterial suspensions, using two consecutive TFF steps (Tangential Flow Filtration steps: micro-filtration at 0.22 μm and thereafter a second micro-filtration at 0.1 μm) at the end of the fermentation process [104,114,129,154].

Release of GMMA is highly dependent on the envelope structure. Defect in proteins linkage the outer membrane to peptidoglycan layer or involved in a structural network between the inner, outer membranes and peptidoglycan layer result in the shedding of large amounts of GMMA.

The Tol-Pal system is present in most of Gram-negative bacteria, it is required to maintain outer membrane integrity, a mutation in any of tol-pal genes confer a defect in other membrane integrity resulting in a major release of outer membrane GMMA [123,129,144,145,147,155].

NVGH, to over produce GMMA, is developing mutant strain deleting the *tolR* gene, which result in substantial overproduction of outer membrane particles without loss of membrane integrity [129,156].

NVGH has developed another innovative platform, describing a simplified methodology for extraction and purification of the O-antigen core portion of *Salmonella* lipopolysaccharide, suitable for large-scale production. Lipopolysaccharide extraction and delipidation are performed by acetic acid hydrolysis of whole bacterial culture and can take place directly in a bioreactor, without previous isolation and inactivation of bacteria. Further O-antigen core purification consists of rapid filtration and precipitation steps, without using enzymes or hazardous chemicals. The process was successfully applied to various *Salmonella enterica* serovars (*paratyphi* A, Typhimurium, and *enteritidis*), obtaining good yields of high-quality material, suitable for conjugate vaccine preparations [157].

III. PhD PROJECT AIM

The purpose of my PhD project was to investigate the possibility to develop low cost vaccines applicable to Gram-negative strains, intended for neglected infectious diseases of impoverished communities, using the GMMA technology platform and the acid hydrolysis approach.

This work showed the possibility to use GMMA particles as starting material for membrane components extraction, optimizing new and innovative platform developed according to the membrane component target nature, LPS or proteins, in order to decrease the costs of goods (COGs).

The present work is focused on the O: 4,5-antigen polysaccharide extraction, purification and characterization from *Salmonella* Typhimurium SL1344 *tolR*⁻ GMMA.

Following are shown the results obtaining from the O: 4,5-antigen molecules extracted and purified from *Salmonella* Typhimurium SL1344 *tolR*⁻ biomass, *Salmonella* Typhimurium SL1344 *tolR*⁻ GMMA and *Salmonella* Typhimurium SL1344 Wild type biomass.

The O: 4,5-antigen polysaccharide from GMMA, released by *Salmonella* Typhimurium SL1344 *tolR*⁻, was further characterized and compared with the *Salmonella* Typhimurium SL1344 Wild type O: 4,5-antigen structure.

The electron microscopy approach was applied to study GMMA surface and particles interactions.

IV. MATERIALS AND METHODS

1. Bacterial strains

Salmonella Typhimurium SL 1344 was provided by the Wellcome Trust Institute (NCBI Ref Seq: NC_003197.1).

The *tolR* of *Salmonella* Typhimurium SL 1344 was generated using a pAJD434 plasmid and the *tolR* gene was replaced with a *Kan* cassette encoding kanamycin resistance.

The upstream and downstream regions of *tolR* were amplified by PCR with primers *tolR*1 (CTAGTCTAGAGCCATCATTATTCCAGCGAAC) and *tolR* (AGCTTGATATCGGCTTACCCCTTGTTGCTTTC) for the upstream region and *tolR*3 (AGCTTGATATCAGTCTGCGTCCCGTTGGCTT) and *tolR*4 (CCGGGTACCGTTGCGCAGTTTTGCCGCT) for the downstream region, which contained restriction site at the 5' and 3' end (XbaI and EcoRV for the upstream and EcoRV and KpnI for the downstream region), to generate ~500-base pair (pb) long DNA fragments. These fragments were digested with the respective restriction enzyme (RE) and ligated to a previously XbaI and kpnI-digested pBlueScript18 (pBS) (quiagen) to generate a pBS containing 500pb upstream and downstream regions of *tolR* with a restriction site of EcoRV in the middle. This plasmid was digested with EcoRV and ligated to a kanamycin resistance cassette (*kan*^r) previously amplified from pUC4K with primers *tolR*5 (AGCTTGATATCAAAGCCACGTTGTGTCTCAAAATCTC) and *tolR*6 (AGCTTGATATCTGAGGTCTGCCTCGTGAAGAAG), corresponding to the same EcoRV sites at both the 5' and 3' end, and digested with RE, to generate a plasmid containing the 500bp *tolR* upstream region, the *kan*^r cassette and the 500 bp *tolR* downstream region (pBS *tolR* 5' *kan*^r *tolR*3'). This plasmid was linearized with an RE and linear DNA fragment was amplified by PCR with primers *tolR*1 and *tolR*4 to yield the DNA fragment *tolR* 5' *kan*^r *tolR*3'. This fragment was electroporated into S.Typhimurium SL1344 expressing the phage λ red homologous recombination system encoded by plasmid pAJD434, and mutants where *tolR* gen was replaced with the *kan*^r resistance were selected on Luria-Bertani (LB) plates containing 25 μ g/ml kanamycin. Gene replacement was confirmed by PCR amplification of the genomic DNA of the mutant colonies using

primers *tolR7* (CGCCGCTGCGCGTGAGGCGG) and *tolR8* (CCGCTTTCTTCGCGTCC) annealing upstream and downstream of *tolR*.

2. Liquid growth media

A. Complex media

The complex media composition was: 30 g/L Yeast extract (BD, BD243621), 5 g/L KH_2PO_4 (Sigma, P5655), 20g/L K_2HPO_4 (Sigma, P2222), 1M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma, 63138), 5g/L Glycerol (Carlo Erba, 346161).

B. Chemical defined media

The Chemically Defined Media (CDM) composition was: 13.3 g/L KH_2PO_4 (Sigma, P5655), 4 g/L $(\text{NH}_4)_2\text{HPO}_4$ (Sigma, P215996), 1.7 g/L citric acid monohydrate (Sigma, C1909), 1M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma, 63138), 1 mL/L Trace Elements, 1mL/L Vitamins and 3% Glycerol (Carlo Erba 346161), pH 6.7. Trace Elements solution contained: 2.5 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (Sigma, 15862), 15 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (Sigma, M8054), 1.5 g/L $\text{CuCl}_2 \cdot \text{H}_2\text{O}$ (Sigma, 307483), 3 g/L H_3BO_3 (Sigma, B9645), 2.5 g/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (Sigma, M1651), 2.5 g/L $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$ (Sigma, 383058) and 100 mM ferric citrate (Sigma, F3388). Vitamins solution contained: 0, 05 g/L thiamine (Sigma, T90344), 0, 01 g/L nicotinic acid (Sigma, N0761), 0, 01 g/L Ca-pantothenate (Sigma, C8731) and 0, 01 g/L Pyridoxine-HCl (Sigma, P6280), 0,001g/L B12 (Sigma, C3607).

Glycerol was used as carbon source: 5g/L for shaker flask culture and 30g/L for fermentation process.

Defined media could be supplemented with amino acids, 0.1g/L each: L-alanine (Sigma, A7469), L-arginine (Sigma, A8094), L-aspartic acid (Sigma, A7219), L-asparagine (Sigma, A4159), L- cysteine (Sigma, C7352), L-glutamic (Sigma, G8415), L-Glutamine (Sigma, G8540), L-histidine (Sigma, H6034), L-isoleucine (Sigma, I7403), L-leucine (Sigma, L8912), L-lysine (Sigma, L5501), L-Methionine (Sigma, M5308), L-phenylalanine (Sigma, P5482), L-proline (Sigma, P5607), L-serine (Sigma, S4311), L-threonine (Sigma, T8441), L-tryptophan (Sigma, T8941), L-tyrosine (Sigma, T8566), L-valine (Sigma, V0513).

C. Growth conditions in shaker flask

Salmonella Typhimurium SL1344 wild type and *Salmonella* Typhimurium SL1344 *tolR*⁻ were grown in shaker flasks (Corning, 431401), in 150 mL of complex medium, at 37°C (Thermo Scientific MaxQ 8000 stackable shakers) and shaking at 200 rpm.

Salmonella Typhimurium SL1344 *tolR*⁻ was grown in 125mL shaker flask (Corning, 430421) using 30mL of chemical defined medium, 37°C, overnight and shaking at 200 rpm.

Salmonella Typhimurium SL1344 *tolR*⁻ was grown in 500mL shaker flask (Corning, 431401) using 150mL of chemical defined medium, 37°C overnight and shaking at 200 rpm. Defined medium was supplemented with L-histidine 0.1g/l, 0.3g/L, 0.6g/L.

The cultures optical density (OD) was measured at 600 nm wavelength (Implen, OD600 photometer)

3. Fermentation conditions

S. Typhimurium SL1344 wild type and *S. Typhimurium* SL1344 *tolR*⁻ were grown, up to 30 OD, in 3L of chemically defined medium, using a 5L working volume fermenter (Sartorius B-plus).

Temperature was maintained at 37°C, pH was fixed at 6.7 set point and controlled with 28% NH₄OH (Sigma, 338818), oxygen saturation was set at 30% and maintained by agitation controller.

Glycerol (30g/L) was used as carbon source and additional medium feedings with histidine (0,2g/L) were done.

Antifoam polipropilenglicole (PPG) 0.25 g/L was added at the beginning of the fermentation process.

The cultures optical density (OD) was measured at 600 nm wavelength (Implen OD600 photometer).

4. TFF cassettes Pore size screening for GMMA recovery

3L of *S. Typhimurium* SL1344 *tolR* fermentation broth were processed through the 0.2 μm cut off Tangential Flow Filtration (TFF) membrane (Sartorius, Sartocan Slice, Hydrosart, 0.1 m^2 , 3051860701W) to divide bacteria biomass, in the retentate, form GMMA particles in the permeate, obtaining the “GMMA 0.2 μm permeate” sample.

1L of “GMMA 0.2 μm permeate” was concentrate ten time (up to 100mL) using the 0.1 μm pore size TFF cassette (Sartorius, Sartocan Slice 200, PESU 0.02 m^2 , 3081545802W). The resulting retentate was washed with ten diafiltration steps, against PBS, maintaining the same volume and then collected obtaining the “GMMA 0.1 μm retentate” sample.

The permeate at the end of the 0.1 μm TFF filtration step and the ten diafiltrations volumes were combined in a unique sample and subjected to a further TFF concentration step (up to 100mL) using the 500 KDa membrane cassette (Pall, Centramate 0.019 m^2 , suspended screen channel, cod. 0S500C11P2). Sample was washed by ten diafiltration steps against PBS, maintaining the same volume and at, the end, the retentate was collected obtaining the “GMMA 500KDa retentate” sample.

The 500kDa TFF permeate and the diafiltrations volumes were combined and subjected to another TFF concentration step (up to 100mL) through the 100KDa TFF cassette (Sartorius, Sartocan Slice 200, Hydrosart, 0.02 m^2 , 3081446802E). Sample was washed by ten diafiltration steps against PBS maintaining the same volume., at the end the retentate was collected obtaining the “GMMA 100KDa retentate” sample.

The permeate resulting after the 100KDa TFF was subjected to another TFF concentration step, (up to 100mL) through the 30KDa TFF cassette (Sartorius, Sartocan Slice 200, Hydrosart, 0.02 m^2 , 3081445902E) without diafiltration steps. The resulting retentate and permeate were both collected (fig.1).

All samples were sterilized using the 0.22 μm filters (Corning, 431154) and stored at 4°C.

20mL of all samples, at the end of each step, were ultracentrifuged for 2h, at 180000g and 4°C (Beckman coulter, L-90K, 45Ti-rotor). The resulting pellets were resuspended in 5 ml of PBS.

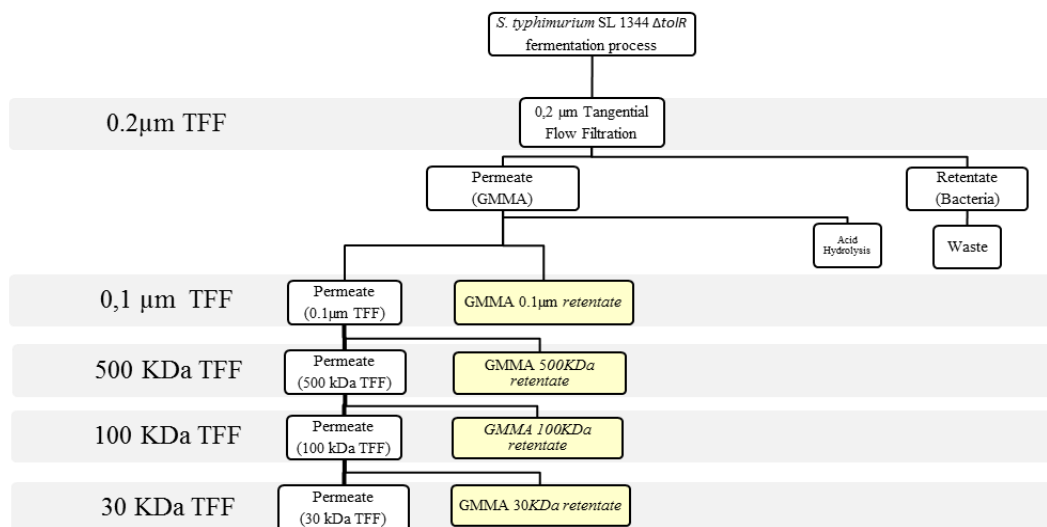


Figure 1: TFF cassettes Pore size screening for GMMA recovery from *Salmonella Typhimurium* SL1344 $\Delta tolR$. Experimental design

5. LPS extraction using the hot phenol method

A. Samples preparation

a) *Salmonella Typhimurium* SL1344 wild type

50 ml of *Salmonella Typhimurium* SL1344 wild type bacterial culture, grew up to 20 OD in shaker flask (Corning, 431401) and in complex medium, were centrifuged for 20 minutes, at 4000g and 4°C (Allegra X-15R, Beckman Coulter). The resulting pellet was resuspended in 2ml of PBS (Phosphate Buffer Saline) to reconstitute the “Wild type BIOMASS Samples” (Figure 2).

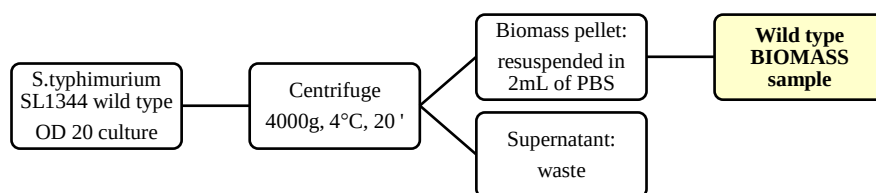


Figure 2: Scheme of sample preparation from *Salmonella typhimurium* SL1344 wild type culture

b) Salmonella Typhimurium SL1344 tolR⁻ Biomass

100 ml of *Salmonella Typhimurium* SL1344 *tolR⁻* bacterial culture, grew up to 20 OD in shaker flask (Corning, 431401) and in complex medium, were splitted in two 50 mL Falcon-tubes and centrifuged for 20 minutes at 4000g and 4°C (Allegra X-15R, Beckman Coulter). The resulting pellet from one tube was resuspended in 2ml of PBS to reconstitute the “*tolR⁻ BIOMASS sample*” and the other one was resuspended in 1 ml of PBS to reconstitute the “*tolR⁻ TOTAL sample*” as descibed following (Fig. 3).

c) Salmonella Typhimurium SL1344 tolR⁻ GMMA

The supernatant after the centrifugation of 100 mL of *Salmonella Typhimurium* SL1344 *tolR⁻* Bacterial cultures, at 20 OD, was divided in two high speed centrifuge tubes (50mL each) and spinned down by ultracentrifugation for 2 hours, at 180000g and 4°C (Optima L 90K Beckman Coulter, 45Ti-rotor). One of the resulting pellet was resuspended in 2 ml of PBS to reconstitute the “*tolR⁻ GMMA sample*” and the other one was resuspended in 1 ml of PBS and then mixed with the sample prepared before to constitute the “*tolR⁻ TOTAL sample*” (Fig.3)

d) *Salmonella Typhimurium* SL1344 *tolR*-TOTAL: GMMA + BIOMASS

The TOTAL sample was reconstituted combining 1ml *tolR*-BIOMASS sample with 1ml *tolR*-GMMA sample (Fig.3).

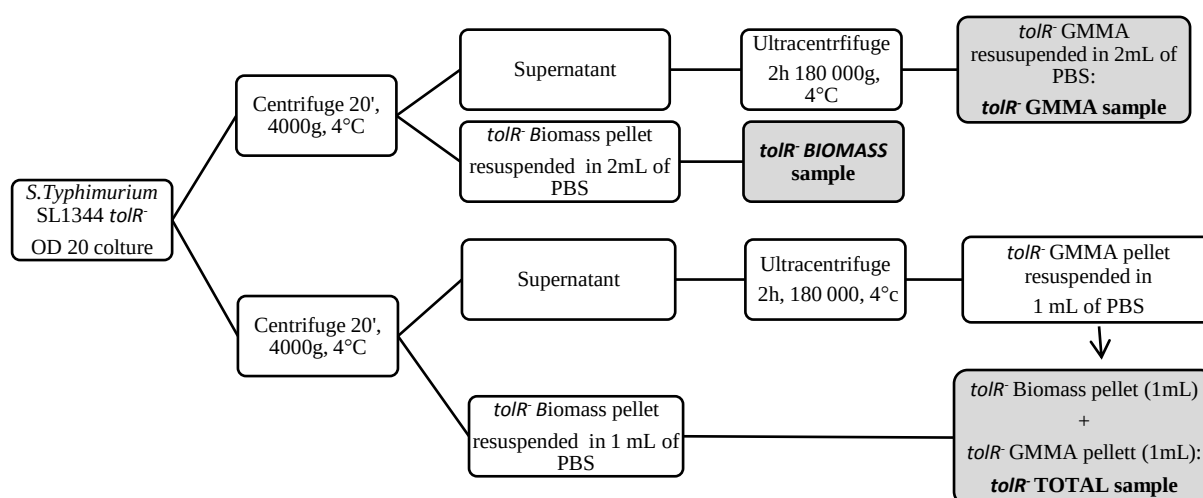


Figure 3. Scheme of samples preparation from *Salmonella typhimurium* SL1344 *tolR*-culture

B. Hot Phenol extraction of LPS

500 µL of all samples were boiled 30-40 minutes in the thermo block at 100°C.

1µg/uL of ProteinaseK (Promega, V302) was added in each sample and incubated at 60 °C overnight.

An equal volume (500 µL) of saturated phenol solution at pH 8.0 (Sigma, P4557) was added and incubated for 3h at 70°C with occasional mixing. At the end, samples were centrifuged 90 minutes, 15000g and 2°C (Allegra X-22R, Beckman Coulter).

The top water phase was recovered and 2 volumes of 100% ethanol (BDH Prolabo, 20821) were added.

Samples were placed at -80°C for 90 minutes, to let the polysaccharide to precipitate.

Samples were centrifuged 1h, 15000g and 4°C (Allegra X-22R, Beckman Coulter).

The final pellets were lyophilized overnight and then resuspended, in the same initial volume of Milli-Q water.

C. Acidic hydrolysis of LPS extracted using the Hot Phenol method

1% Acetic acid (Carlo Erba, 302011) was added to the LPS samples, extracted as described before, to reach pH 4-4, 5 and thereafter were placed in a thermo block 90 minutes at 100°C.

The solution was then neutralized by adding 28% NH₄OH (Sigma, 338818) to reach a pH value of 6-7.

Samples were centrifuged overnight at 15000xg and 4°C (Allegra X-22R, Beckman Coulter). Supernatants were collected and dialyzed, against water, using Slide-A-Lyzer with a 7 KDa cut-off membrane (Slide-A-Lyzer Dialysis Cassettes, 7K MWCO Thermofisher 66373).

6. Direct acid hydrolysis on bacteria or GMMA samples for O-antigen release into the supernatant

1% Acetic acid (Carlo Erba, 302011) was added directly to the sample (bacteria or GMMA samples) to reach pH 4-4, 5 and heating to 100°C for 6h. The hydrolysis was performed at 2mL scale in 4mL Wheaton glass vials, using the boiling water bath. The hydrolysis, at 2L scale, was performed using a heat-jacketed glass round- bottom flask connected to a condenser. The resulting solution was neutralized adding 28% NH₄OH (Sigma, 338818) to reach a pH value of 6-7.

7. O:4.5-antigen purification process from *S. Typhimurium* SL1344 wild type

S. Typhimurium SL1344 wild type fermentation broth, at the end of the hydrolysis, was passed through a 0.2 μm TFF membrane (Sartorius, Sartocan Slice Hydrosart , 0.1 m^2 , 3051860701W) (Figure 4).

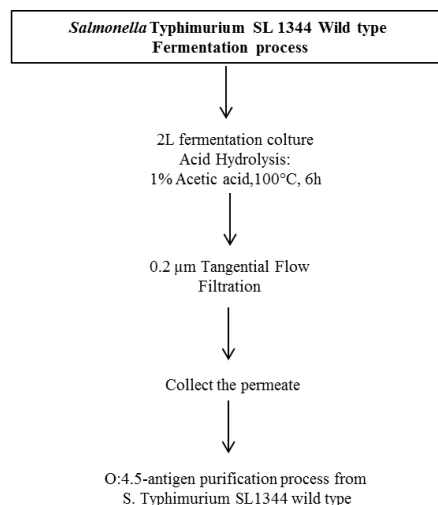


Figure 4. Flow chart for O-antigen extraction from *S. Typhimurium* SL1344 wild type

S. Typhimurium SL1344 wild type was directly hydrolyzed at the end of the fermentation process and then pass through 0.2 TFF membrane, to divide the residual biomass from the permeate containing O-antigen..

2L of the resulting permeate were concentrated 10-folds by Tangential Flow Filtration using the 30KDa cut-off filter cassette (Sartorius, Sartocan Slice 200 Hydrosart, 0.02 m^2 , 3081445902E). Twenty diafiltration, maintaining the same volume, were done to wash the sample (ten against 1 M NaCl and ten against water). The first 30KDa TFF retentate was collected.

Citrate buffer (200 mM, pH 2.7) was added to 50mL of the first 30 KDa TFF sample, to give a final concentration of 20 mM, pH 3. The solution was mixed at room temperature for 30 min, further centrifuged (Beckman Allegra X-15R) for 30 min, 4000g at 4°C and the resulting pellet was discarded.

The supernatant, containing O-antigen, was further purified by negative cationic exchange chromatography, using a Sartobind S MA75 filter (Sartorius) equilibrated with 20 mM citrate buffer at pH 3.

The flow-through, containing O-antigen, was collected and 500 mM Na₂HPO₄ (Sigma, S0876), absolute EtOH (BDH Prolabo 20821) and 5 M CaCl₂ (Sigma-C8106) were added, to give final concentrations of 25 mM Na₂HPO₄, 24% EtOH (v/v) and 200 mM CaCl₂. The solution was mixed at room temperature for 30 min, centrifuged (Beckman Allegra X-15R) at 4000g, 4°C for 30 min, and the pellet was discarded.

The resulting supernatant was passed through the PD-10 desalting column (GE Healthcare Life Sciences), in order to exchange the buffer with water, and analyzed.

8. Comparison of four different O: 4,5-antigen purification processes starting from GMMA produced by *S. Typhimurium* SL1344 *tolR*-

2L “GMMA 0.2µm permeate” sample, from *Salmonella* Typhimurium SL1344 *tolR* fermentation broth processed with 0.2 µm TFF membrane, were treated with the acid hydrolysis approach (Fig.5). At the end of the hydrolysis this sample was centrifuged for 20minuts, at 8000g, at 4°C (Beckman Coulter Avanti J-26 XP Centrifuge, JA-10 Fixed Angle Rotor), to divide the residual membrane fragments to the supernatant containing O-antigen.

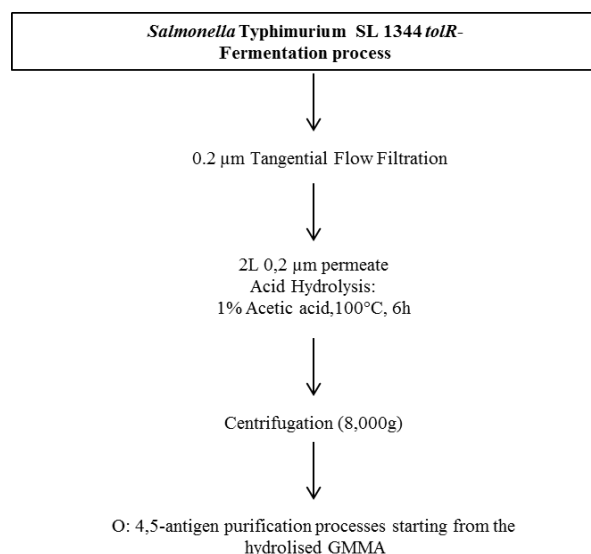


Figure 5. Flow chart for O-antigen extraction from GMMA produced by *S. Typhimurium* SL1344 *tolR*-.

The hydrolyzed GMMA material was concentrated 10-folds by TFF using the 30KDa cut-off filter cassette (Sartorius, Sartocoon Slice 200 Hydrosart, 0.02 m², 3081445902E). Twenty diafiltration, maintaining the same volume, were done to wash the sample (ten against 1 M NaCl and ten against water). The “first 30KDa retentate” sample was collected and used to test different purification schemes described ahead and summarized in the in Fig. 6

9. Complete purification process for O:4.5-antigen

50 ml of the *first 30KDa retentate*” was subjected to the same purification protocol used to purify the O:4.5-antigen from the biomass of *S. Typhimurium* SL1344 wild type biomass(Figure 6a).

A. Precipitation with Citrate buffer followed by cationic exchange chromatography

Citrate buffer (200 mM, pH 2.7) was added to 50 ml of the *first 30KDa retentate* to give a final concentration of 20 mM, pH 3. The resulting solution was mixed at room temperature for 30 min. At the end of the mixing time, the solution was centrifuged (Beckman Allegra X-15R) for 30 min, 4000g, at 4°C. The pellet was discarded while the supernatant was collected and passed through the chromatographic filter Sartobind S MA75 filter (Sartorius), previously equilibrated with the same buffer (Citrate buffer 20 mM, pH 2.7).

The filter flow through was collected and passed through the PD-10 desalting column (GE Healthcare Life Sciences), in order to exchange buffer with water, and analyzed.

B. Precipitation with Citrate buffer followed by CaCl₂/EtOH precipitation

Citrate buffer (200 mM, pH 2.7) was added to 50 ml of the *first 30KDa retentate* to give a final concentration of 20 mM, pH 3. The resulting solution was mixed at room temperature for 30 min. At the end of the mixing time, the solution was centrifuged (Beckman Allegra X-15R) for 30 min, 4000g, at 4°C. The pellet was discarded while the supernatant was collected and added with 500 mM Na₂HPO₄, absolute EtOH and 5 M CaCl₂ to give final concentrations of

25 mM Na_2HPO_4 , 24% EtOH (v/v) and 200 mM CaCl_2 . The solution was mixed at room temperature for 30 min, centrifuged (Beckman Allegra X-15R) at 4000g, 4°C for 30 min and the pellet was discarded; the supernatant was collected, passed through a PD-10 desalting column (GE Healthcare Life Sciences) and analyzed (Figure 6c).

C. Cationic exchange chromatography

Citrate buffer (200 mM, pH 2.7) was added to 50 ml of the *first 30KDa retentate* to give a final concentration of 20 mM, pH 3. The resulting solution was immediately passed through Sartobind S MA75 filter (Sartorius), already conditioned with the same buffer (Citrate buffer 20 mM, pH 2.7). The flow-through was passed through a PD-10 desalting column (GE Healthcare Life Sciences) and analyzed (Figure 6d).

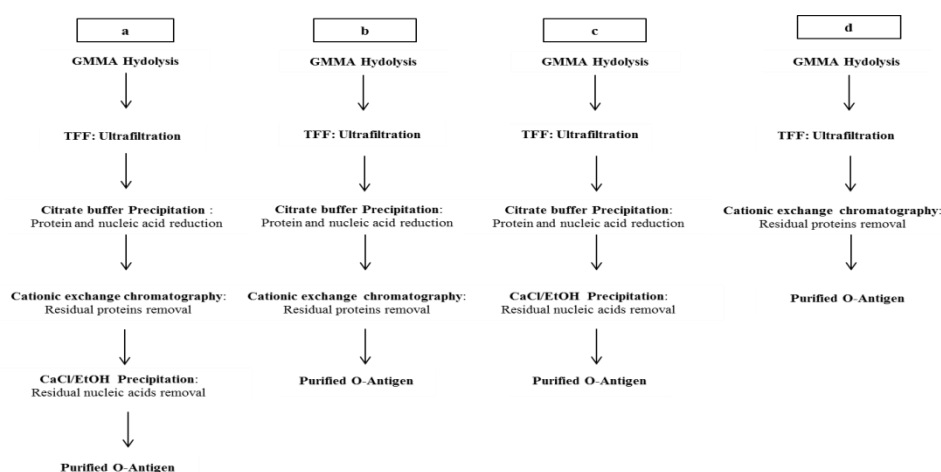


Figure 6. Four different schemes of purification process for the O:4.5-antigen, starting from GMMA produced by *S.typhimurium* SL1344 *tolR*⁻; a) O:4.5-antigen purified using the standard purification protocol, developed starting from the biomass; b) Only the first precipitation step and the cationic exchange chromatography were tested; c) Precipitations with Citrate buffer and $\text{CaCl}_2/\text{EtOH}$ were tested avoiding the *cationic exchange chromatography*; d) Only the cationic exchange chromatography was tested

10. Determination of the Total Sugar Content in Samples by the Phenol-Sulfuric Assay

Quantification of total sugar concentration in each sample was determined using the Phenol Sulfuric Acid Colorimetric Assay [158]

Reagents:

- 5% (w/v) Phenol solution (Sigma, P5566)
- H₂SO₄ (Sigma, 84720)
- 0.5 mg/mL Glucose solution (Sigma, 49158).

In order to quantify the total sugar amount, sample concentration must be in the range of calibration curve, 62.5 - 250 µg/mL of Glucose. The calibration curve was made starting from 0.5 mg/mL of Glucose standard solution. Each sample dilution (200 µL) was prepared in duplicate and 200 µL of water was used as blank solution.

200 µL of Phenol solution and 1 mL of H₂SO₄ concentrated solution were added consecutively to each sample (blank solution, calibration curve, sample dilutions). All samples were incubated 10 minutes at room temperature, under chemical hood. Thereafter samples were mixed by Vortex and then incubated for further 30 minutes. At the end of the incubation time, samples were read at 490nm (Spectrophotometer Perkin Elmer Lambda 25).

11. Determination of Protein Content by Micro BCA Protein Assay

Protein content was measured by the Micro BCA Method (Thermo scientific Micro BCA Protein Assay Kit), following the manufacturer's instructions.

12. Estimation of DNA content by absorption at 260nm

An estimation of µg/mL DNA concentration can be obtained multiplying by 50 the absorption value obtained reading the sample at 260 nm (Spectrophotometer: Perkin Elmer Lambda 25).

The Nucleic acids content was estimated measuring the absorbance at 260 nm, the maximum absorption wavelength of DNA. An overestimation could occur due to the presences of proteins or peptides, which absorbs at the same wavelength.

13. Size exclusion chromatography

A. TSK gel 3000 PWXL

In order to estimate the molecular size distribution of O-antigen populations, samples were analyzed by HPLC- Size Exclusion Chromatography, using a TSK gel 3000 PWXL column (30 cm× 7.8 mm; particle size 7 µm, Tosoh Bioscience, 808021)

The mobile phase was 100 mM NaCl (Sigma, S9888), 100 mM NaH₂PO₄ (Sigma, S8282), 5% CH₃CN (Prolabo; 20060.320) pH 7 and the flow rate was of 0.5 mL/min.

The refractive index detector was used to detect the O-antigens size distributions.

The UV detector (A₂₆₀) and fluorescence detector (ex280/em336 nm) were used to reveal peaks corresponding to impurities, like proteins and nucleic acids.

B. Size exclusion chromatography on Sephacryl S-100 HR 16/60

The purified O-antigen solution was loaded on Sephacryl S-100 HR 16/60 Gel Filtration column (16 x 600 mm; particle size 25 µm-75 µm; GE health care life sciences) using 10mM NaH₂PO₄, pH 7.2 as running buffer and a flow rate of 0.5 mL/min.

14. HPAEC-PAD

Rhamnose (Rha), galactose (Gal), glucose (Glc) and mannose (Man) are present once in each *S. typhimurium* O-antigen repeating unit, and N-acetyl glucosamine (GlcNAc), is present only in the core region. Their content in the purified O-antigen solution was estimated by HPAEC-PAD after the monosaccharides release due to the acid hydrolysis. Commercial monomer sugars were used for preparation of calibration curves, for Rha (Fluka, 83650), Gal (Sigma, 48259), Glc (Sigma, 49158) and Man (Sigma, 63580) quantification. O-antigen samples were

diluted to have each sugar monomer in the range 0.5-10 µg/mL , were hydrolyzed at 100°C for 4 h in 2 M TFA(Sigma, 302031), conditions found to be optimal for release of all monomers without degradation. For GlcNAc quantification, O-antigen samples, diluted to have a GlcNAc (Sigma, a8625) concentration in the range 0.5-10 µg/mL, were hydrolyzed at 100°C for 6 h in 1 M TFA, then cooled to 2-8°C for about 30 min and, dried using a vacuum centrifuge (SpeedVac) overnight. The following day they were, reconstituted in water and filtered using 0.45 µm Acrodisc (PALL) filters before chromatographic analysis.

HPAEC-PAD was performed using a Dionex ICS3000 instrument equipped with a CarboPac PA10 column (4x250 mm) coupled with PA10 guard column (4x50 mm). Separation of the sugars was performed with a flow rate of 1 mL/min eluting in a gradient from 10 mM NaOH to 18 mM NaOH over 20 min. After washing for 20 min with 100 mM AcONa in 28 mM NaOH, the column was re-equilibrated with 10 mM NaOH for 20 min. The effluent was monitored using an electrochemical detector in the pulse amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. The Dionex standard quadruple-potential waveform for carbohydrates was used. The resulting chromatographic data were processed using Chromeleon software 6.8. Calibration curves were built for each sugar monomer (0.5-10 µg/mL). The standards were hydrolyzed and analyzed in the same way as samples. For GlcNAc, glucosamine (GlcN) was the species detected by HPAEC-PAD after hydrolysis.

15. ELECTRON MICROSCOPY

A. Samples preparations

S. Typhimurium SL1344 *tolR*⁻ was fermented up to 30 OD as described before.

A small volume (100 µL) of fermentation broth of *S. Typhimurium* SL1344 *tolR*⁻ was collected and directly used to prepare the sample for Transmission Electron Microscopy (TEM) analysis. At the same time, 50 mL of the same fermentation broth of were centrifuged 20 minutes at 4000g at 4°C (Allegra X-15R, Beckman Coulter) to divide the biomass from the supernatant containing GMMA.

The pellet obtained from biomass of *S. Typhimurium* SL1344 *tolR*⁻ was resuspended in 50 mL of PBS, 100 µL of this preparation were collected and prepared for TEM analysis.

The Supernatant containing GMMA was ultra-centrifuged for 2 hours, at 180000g and 4°C (Optima L 90K Beckman Coulter, 45Ti-rotor). The resulting GMMA pellet was resuspended in 2 mL of PBS, obtaining the sample “GMMA before the 0.2 µm TFF”, 100 µL of this GMMA sample were treated for TEM analysis.

B. Negative staining of GMMA

Droplets of GMME suspensions (10 µL) were placed on formvar-carbon coated grids and allowed to adsorb for 60 sec. Excess liquid was removed gently touching the filter paper. The adsorbed specimen was then fixed for 2 min at room temperature floating on a drop of 4% paraformaldehyde plus 0.05% glutaraldehyde in PBS, pH 7.2. Negative-staining was performed by first washing the specimen grid on a drop of negative stain (2% uranyl acetate in distilled water), blotting and repeating this step once more, this time leaving the specimen grid for 60 sec on a new drop of negative stain solution.

Samples were observed at a JEOL 1200 EX II electron microscope. Micrographs were acquired by the Olympus SIS VELETA CCD camera equipped the iTEM software.

C. Transmission Electron Microscopy (TEM) of GMMA and bacteria

For Transmission Electron Microscopy (TEM), samples were pre-fixed for 20 min at 4°C with 2% paraformaldehyde + 2.5% glutaraldehyde in cacodylate sucrose buffer (0,1 M cacodylate, 0,09 M sucrose, 0,01 M CaCl₂, 0,01 M MgCl₂, pH 6,9) containing 0,075% ruthenium red and 0,075 M lysine acetate. After washing two changes for 30 min each at 4°C in the same buffer containing 0,075% ruthenium red, they were fixed with 2% paraformaldehyde+2,5% glutaraldehyde in cacodylate sucrose buffer containing 0,075% ruthenium red overnight at 4°C. They were washed again, three changes for 30 min each at 4°C in the same buffer, and post-fixed with 2% osmium tetroxide in cacodylate buffer containing 0.075% ruthenium red for 1h at RT. Specimens were washed in the same buffer, three changes for 30 min each at 4°C and dehydrated in a graded ethanol series. Then samples were infiltrated for two days with

mixtures of LRWhite resin/ethanol in different percentages. At the end of the procedure, samples were embedded in LRWhite resin. Embedded material was cut with Reichert Ultracut ultramicrotome using a diamond knife. Thin sections (60-80 nm) were collected on copper grids, stained with uranyl acetate and lead citrate, and observed with a JEOL 1200 EX II electron microscope. Micrographs were acquired by the Olympus SIS VELETA CCD camera equipped the iTEM software

V. RESULTS

In this study *Salmonella* Typhimurium SL1344 was chosen for production of generalized modules for membrane antigens (GMMA). The mutation of the *tolR* gene, that is part of the Tol-Pal system, important for the stability and integrity of the outer membrane, has been shown to increase the release of GMMA from the surface of other Gram negative bacteria into the culture medium.

In this chapter, we describe the optimization of growth conditions for *S. Typhimurium* SL1344 *tolR*⁻ strain to overproduce GMMA, released in the fermentation broth, and further purified, using different approaches.

We show the possibility to use GMMA as O:4,5 -antigen extraction source, proposing an alternative purification protocol based on the GMMA characteristics and properties.

We also characterize the GMMA O:4,5-antigen structure to ensure the O:4,5 -antigen equivalence from GMMA with the O:4,5 -antigen from *S. Typhimurium* SL1344 wild type comparing the structure and the sugar composition

1. Chemical defined medium Optimization for *S. Typhimurium* SL1344 *tolR*⁻ strain

In this section we describe the strategies applied to optimize the chemical defined medium for *S. Typhimurium* SL 1344 *tolR*⁻ strain.

To find the best growth condition, *S. Typhimurium* SL 1344 *tolR*⁻ was incubated overnight (37°C, shaking at 200 rpm) in chemical define medium (CDM) supplemented with different amino acids pools, using complex medium as control.

The amino acids pool composition was based on the microbial metabolism and the related capacity to synthesize them starting from the same precursor.

The amino acid pools were prepared in a stock solution, adding 0.1 g/L of each (Table 1).

POOL	AMINO ACIDS COMPOSITION
1	His, Ser, Cys, Gly
2	Trp, Phe, Tyr
3	Asp, Ala, Leu, Ile, Val
4	Asn, Met, Lys, Thr
5	Glu, Gln, Arg, Prol

Table 1. Composition of different amino acids pools.

Pool 1: histidine, serine, cysteine, glycine.

Pool 2: tryptophan, phenylalanine, tyrosine.

Pool 3: aspartate, alanine, leucine, isoleucine, valine.

Pool 4: asparagine, methionine, lysine, threonine .

Pool 5: glutamate, glutamine, arginine, proline.

S. Typhimurium SL 1344 *tolR*⁻, after overnight incubation, was able to growth, from OD 0.04 up to OD 0.26, only in presence of Pool 1 , thus for this strain one or more amino acid present in Pool 1 were necessary (Table 2).

SAMPLES	OD START	OD END
Pool 1	0,04	0,26
Pool 2	0,04	0,04
Pool 3	0,04	0,04
Pool 4	0,04	0,04
Pool 5	0,04	0,04
Complex medium	0,04	10

Table 2. Growth condition in different amino acids pools.

In order to identify the *S. Typhimurium* SL 1344 *tolR*⁻ auxotrophy, we decide to test different pools combination.

As done before, *S. Typhimurium* SL 1344 *tolR*⁻ was grown overnight at 37°C, shaking at 200 rpm, in CDM supplemented with different Pools combination (see Table 3).

POOL COMBINATION	AMINO ACIDS COMPOSITION
1\2	His, Ser, Cys, Gly, Trp, Phe, Tyr
1\3	His, Ser, Cys, Gly Asp, Ala, Leu, Ile, Val
1\4	His, Ser, Cys, Gly Asn, Met, Lys, Thr
1\5	His, Ser, Cys, Gly Glu, Gln, Arg, Prol
2\3	Trp, Phe, Tyr Asp, Ala, Leu, Ile, Val
2\4	Trp, Phe, Tyr Asn, Met, Lys, Thr
2\5	Trp, Phe, Tyr Glu, Gln, Arg, Prol
3\4	Asp, Ala, Leu, Ile, Val Asn, Met, Lys, Thr
3\5	Asp, Ala, Leu, Ile, Val Glu, Gln, Arg, Prol
4\5	Asn, Met, Lys, Thr, Glu, Gln, Arg, Prol

Table 3. Combination of the different amino acids Pools

After 12 hours of incubation, *S. Typhimurium* SL 1344 *tolR*⁻ was able to grow, reaching the same OD value obtained in the Complex Medium, in the Pool 1 amino acids mix (Figure 1).

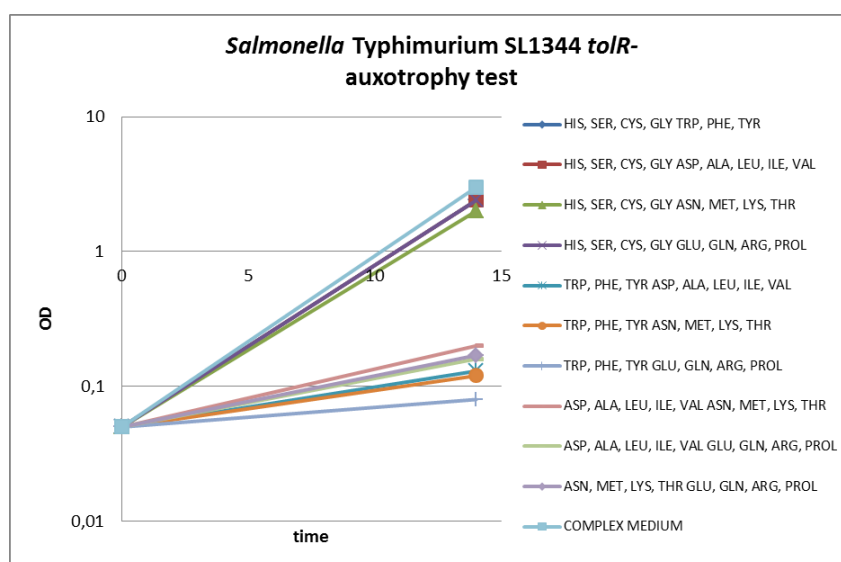


Figure 1. *Salmonella* Typhimurium SL1344 *tolR*⁻ growth curve in different Amino Acid Pool combinations.

This strain showed the ability to grow only in defined media supplemented with Pool 1 amino acids.

In order to identify the specific amino acidic auxotrophy, *S. Typhimurium* SL 1344 *tolR*⁻ was grown overnight, at 37°C and shaking at 200rpm in CDM supplemented with new Pools, obtained from different Pool 1 amino acids combinations, and using complex medium as control.

New stock solutions (0.1 g/L each amino acid) were prepared using: histidine, serine, cysteine, glycine, tryptophan, phenylalanine and tyrosine (Table 4).

POOL RECOMBINATION	AMINO ACIDS COMPOSITION
1R	His, Ser, Cys, ,Trp, Phe, Tyr
2R	His, Ser, Cys, Trp, Tyr
3R	His, Ser, Cys, Phe, Tyr
4R	His, Ser, Cys,Trp, Phe, Tyr
5R	His, Cys, Trp, Tyr
6R	Ser, Cys, Trp, Tyr

Table 4. Composition of different combination of amino acids present in Pool 1.

S. Typhimurium SL1344 *tolR*⁻ growth curve was optimal only with amino acid combinations in which histidine was present, whereas in Pool 6R, composed only of serine, cysteine, tryptophan and tyrosine, bacteria was not able to grow (Figure 2).

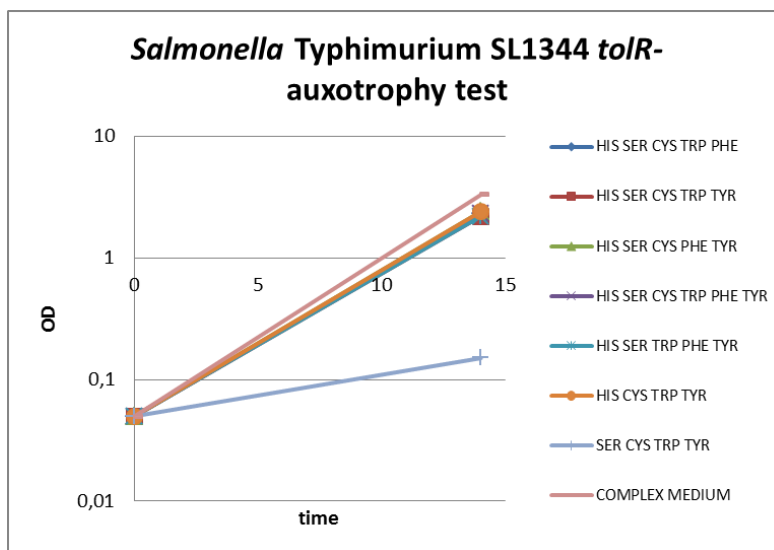


Figure 2. Auxotrophy test, *S. Typhimurium* SL 1344 *tolR*⁻ growth curves in chemically-defined media supplemented with amino acids.

These results showed that, *S. Typhimurium* SL1344 *tolR*⁻ had an histidine auxotrophy.

To create the best *S. Typhimurium* SL1344 *tolR*⁻ growth condition, the CDM was supplemented with different histidine concentrations: 0.1g/L, 0.3g/L, 0.6g/L. *S. Typhimurium* SL1344 *tolR*⁻ was incubated overnight at 37°C, shaking at 200rpm.

At the end of the fermentation time no significant differences were found among the tested conditions (Figure 3).

MEDIUM Composition	STARTING OD	FINAL OD
CDM + 0.1 g/l His	0,05	10
CDM + 0.3 g/l His	0,05	11
CDM + 0.6 g/l His	0,05	13

Figure 3. *Salmonella* Typhimurium SL1344 *tolR*⁻ growth curve in CDM supplemented with different histidine concentrations.

This strain reached similar OD values adding 0.1 g/L, 0.3g/L or 0.6g/L histidine to the CDM. Based on these results, *S. Typhimurium* SL1344 *tolR*⁻ showed an histidine auxotrophy and, for flask scale, the chemical defined medium could be supplemented with 0.1g/L histidine. To verify the scalability and robustness of the process, 3L batch fermentation, in a 5L fermenter, was performed using the CDM supplemented, at the starting point, with 0.1g/L histidine.

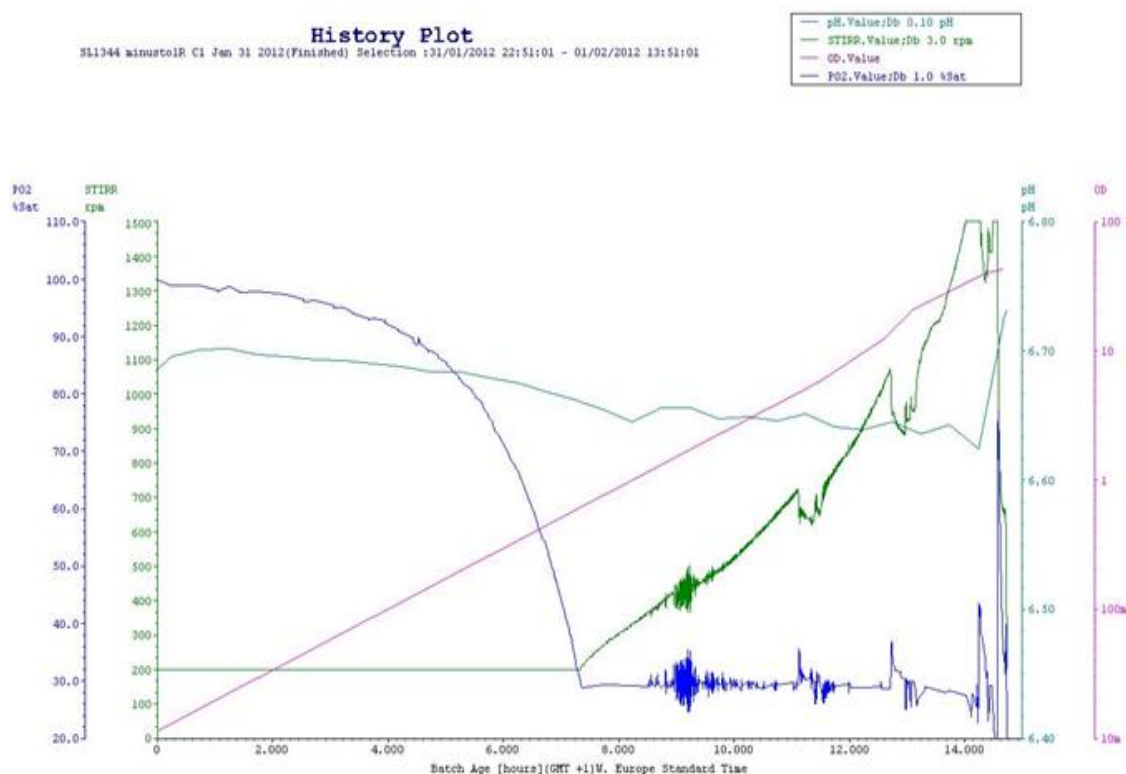


Figure 4. Example of *S. Typhimurium* SL1344 *tolR*⁻ fermentation batch.

At OD 8, OD 16 and OD 30 stirrer decreased and the growth slowed down, this was due to the histidine ending. Feedings of 0.1g/L histidine, were necessary for growth restart. After the last histidine feed, at 15h and 30OD, *S. Typhimurium* SL1344 *tolR*⁻ performed the oxygen spike and the fermentation broth was harvested to collect GMMA.

During the fermentation process, at the 11th hour with OD 8, the stirrer speed decreased and the pO₂ saturation started to increase. This signal is generally associated with the strain suffering due to limiting growth factors, such as nutrient deprivation (Figure 4).

We tried to add 0.1g/L of histidine and immediately after the feeding the stirrer started to increase and the pO₂ saturation level returned up to 30%.

The same situation, during the fermentation process, occurred at the 13th hour with OD 16 and during the 15th hour with 30 OD, and each time 0.1g/L of histidine was added (Figure 4).

Histidine feedings allowed the *S. Typhimurium* SL 1344 *tolR*⁻ to continue to divide.

After the last histidine feed (15th fermentation hour) *S. Typhimurium* SL1344 *tolR*⁻, at 30 OD, produced an oxygen spike, signifying that the carbon source had been exhausted, and harvesting was performed (Figure 4).

At the end of the fermentation process 3 histidine feedings were necessary to reach 30OD and to allow the oxygen spike to occur.

To optimize the fermentation process for *S. Typhimurium* SL1344 *tolR*⁻, it might be possible to add all the histidine required at the beginning of the fermentation process. However, to increase the GMMA production and release it is better to grow the strain under stress conditions, like nutrient deprivation [158,159].

2. O:4,5 Antigen extraction from *S. Typhimurium* SL1344 wild type, *S. Typhimurium* SL1344 *tolR*⁻ and GMMA using the hot phenol method

A. Hot phenol method optimization for high OD bacterial samples or GMMA

Our purpose was to understand if, in GMMA particles, purified from *S. Typhimurium* SL1344 *tolR*⁻, the concentrations of membrane components, such as LPS and proteins, were comparable to those in *S. Typhimurium* SL1344 wild type biomass, when both preparations were grown to the same OD. We chose, as the membrane component target molecule, the O:4,5-antigen.

We used the hot phenol extraction method to purify LPS from GMMA particles and from wild type biomass, and further hydrolyzed the LPS using the acid hydrolysis approach.

Adi Peleg et al. had previously optimized the LPS extraction protocol for culture grown up to OD 1.

We introduced some variations into this protocol for our sample growth at high OD. The first variation, not present in the original protocol, was the preliminary treatment of the samples with Proteinase K in order to degrade membrane proteins (overnight incubation at 60°C).

Another variable that was explored was the phenol incubation time (see materials and methods).

Liquid phenol is an excellent proteins solvent [160] and its role as a deproteinizing agent is well established [161].

In the original protocol, the OD 1 samples were incubated 30 minutes with phenol to permit protein degradation. We tried prolonging the phenol incubation time up to 3h, in order for the protein components of high OD samples to be degraded.

The removal of protein from the bacterial lysate is a crucial step in LPS extraction, and can be performed by the coprecipitation of proteins and nucleic acid in ethanol solution, avoiding the presence of both these contaminants in the final extracted LPS.

We tried the LPS extraction method applying the modified protocol on *S. Typhimurium* SL1344 wild type and *S. Typhimurium* SL1344 *tolR*⁻, grown overnight, in complex medium, at 37°C and 200 rpm shaking, up to 20 OD.

Samples were prepared, in duplicate sets, as described in the Materials and Methods and the LPS hot phenol extraction was performed.

We extracted LPS, from one set of “*S. Typhimurium* SL1344 wild type biomass” sample, “*S. Typhimurium* SL1344 *tolR*⁻ biomass” sample, “*S. Typhimurium* SL1344 *tolR*⁻ GMMA” sample and “*S. Typhimurium* SL1344 *tolR*⁻ TOTAL (biomass+GMMA)” sample.

The other extraction set was used to prepare the “*S. Typhimurium* SL1344 *tolR*⁻ TOTAL 2 (LPS from biomass+ LPS from GMMA)” sample, composed of the LPS extracted from *S. Typhimurium* SL1344 *tolR*⁻ biomass and the LPS extracted from *S. Typhimurium* SL1344 *tolR*⁻GMMA further collected and pooled. This sample was used as control.

The acid hydrolysis approach was applied to all samples (1%Acetic Acid, 100°C, 1h and 30 minutes) and the total sugar amount was quantified by the phenol sulfuric acid method.

S. Typhimurium SL1344 wild type biomass and *S. Typhimurium* SL1344 *tolR*⁻biomass gave a similar sugar concentration (1455 µg/mL vs 1599 µg/mL) (Figure 5).

GMMA from *S. Typhimurium* SL1344 *tolR*⁻ gave, surprising, a good amount of sugar, compared with *S. Typhimurium* SL1344 wild type biomass sample (941 µg/mL vs 1455 µg/mL) (Figure 5).

S. Typhimurium SL1344 *tolR*⁻ TOTAL 2 sample, composed of the combination of LPS extracted from GMMA and LPS from biomass, was expected to have the total sugar concentration consistent with the theoretical sum of single sugar concentrations found in *S. Typhimurium* SL1344 *tolR*⁻ GMMA (941 µg/mL) and in *S. Typhimurium* SL1344 *tolR*⁻ biomass (1599 µg/mL).

S. Typhimurium SL1344 *tolR*⁻ TOTAL 2 gave 2388 µg/mL of total sugar concentration, and this value was indeed comparable with the theoretical sum of LPS from GMMA and LPS from biomass (2540 µg/mL). Thus this sample can be consider our control with respect to the *S. Typhimurium* SL1344 *tolR*⁻ TOTAL sample, in which similar total sugar concentration was expected.

S. Typhimurium SL1344 *tolR*⁻ TOTAL was composed by crude biomass and crude GMMA (see material and methods) and the LPS extraction was performed directly on this crude preparation.

S. Typhimurium SL1344 *tolR*⁻ TOTAL showed a comparable total sugar concentration to *S. Typhimurium* SL1344 *tolR*⁻ TOTAL 2 sample, 2445 µg/mL vs 2388 µg/mL (Figure 5).

Based on these results, we were able to confirm that, the hot phenol extraction method worked well when applied to high culture ODs, rich in protein components, and following the new conditions (samples pretreatment with Proteinase K and prolonged phenol incubation time) that had been tested, the extraction can be performed directly on the total sample composed of crude GMMA and crude biomass.

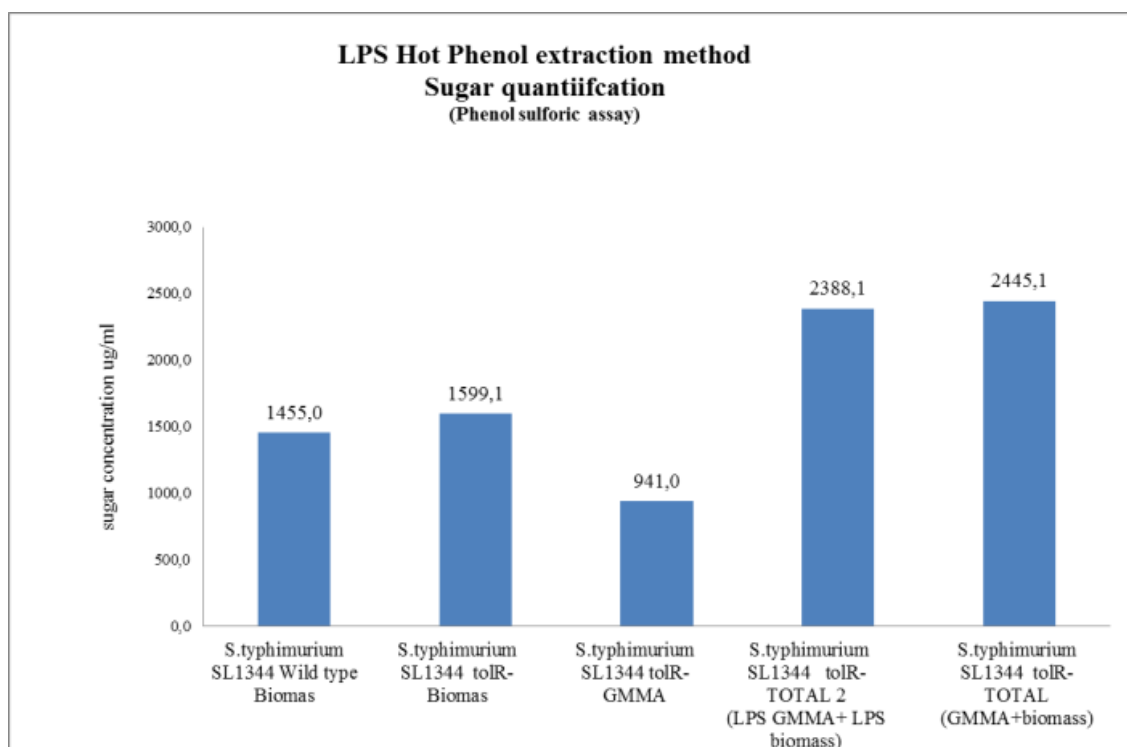


Figure 5. Optimization of the LPS hot phenol method extraction.

LPS from *S. Typhimurium* SL1344 *tolR*⁻ TOTAL 2 sample was used as control. This sample derived from a combination of LPS extracted separately from GMMA and biomass and then mixed. Its concentration was similar to LPS extracted from *S. Typhimurium* SL1344 *tolR*⁻ TOTAL, which is composed of crude GMMA and crude biomass- This indicates that the hot phenol method is effective when directly applied to crude material with a comparable total sugar content.

Total sugar concentration was quantified using the phenol sulfuric assay.

B. O4:5 - antigen purification on the LPS extracted using the hot phenol method from *S. Typhimurium* SL 1344 wild type, *S. Typhimurium* SL1344 *tolR*⁻ and GMMA

In this section we show the results of analysis of O:4,5-antigen from *S. Typhimurium* SL 1344 wild type and *S. Typhimurium* SL1344 *tolR*⁻ to understand the sugar populations present in these two different samples.

LPS was purified using the optimized hot phenol method and further treated with the acid hydrolysis approach.

S. Typhimurium SL1344 wild type and *S. Typhimurium* SL 1344 *tolR*⁻ were growth overnight, in complex medium, at 37°, 200 rpm shaking, up to 20 OD.

Samples were prepared as described in materials and methods.

The O:4,5-antigen portion of the LPS was cleaved, from lipid-A, using the acid hydrolysis approach (1%Acetic Acid, 100°C, 1h and 30minutes) .

Each sample was analyzed in terms of total sugar concentration, using the phenol sulfuric assay, and in terms of O:4,5-antigen population by size exclusion chromatography.

S. Typhimurium SL1344 wild type biomass and *S. Typhimurium* SL1344 *tolR*⁻ biomass showed a similar sugar concentration: SL 1344 wild type biomass contained 1126 µg/mL sugars while SL1344 *tolR*⁻ biomass contained 1111 µg/mL (Figure 6).

Another important confirmation was that, the total sugar amount found in GMMA sample was comparable with the *S. Typhimurium* SL1344 wild type biomass: SL1344 *tolR*⁻ GMMA gave 874 µg/mL LPS vs SL 1344 wild type biomass 1126 µg/mL (Figure 6).

The TOTAL sample, composed by *S. Typhimurium* SL1344 *tolR*⁻ biomass and *S. Typhimurium* SL1344 *tolR*⁻ GMMA, showed the highest total sugar concentration of the various samples, including the *S. Typhimurium* SL1344 wild type biomass.

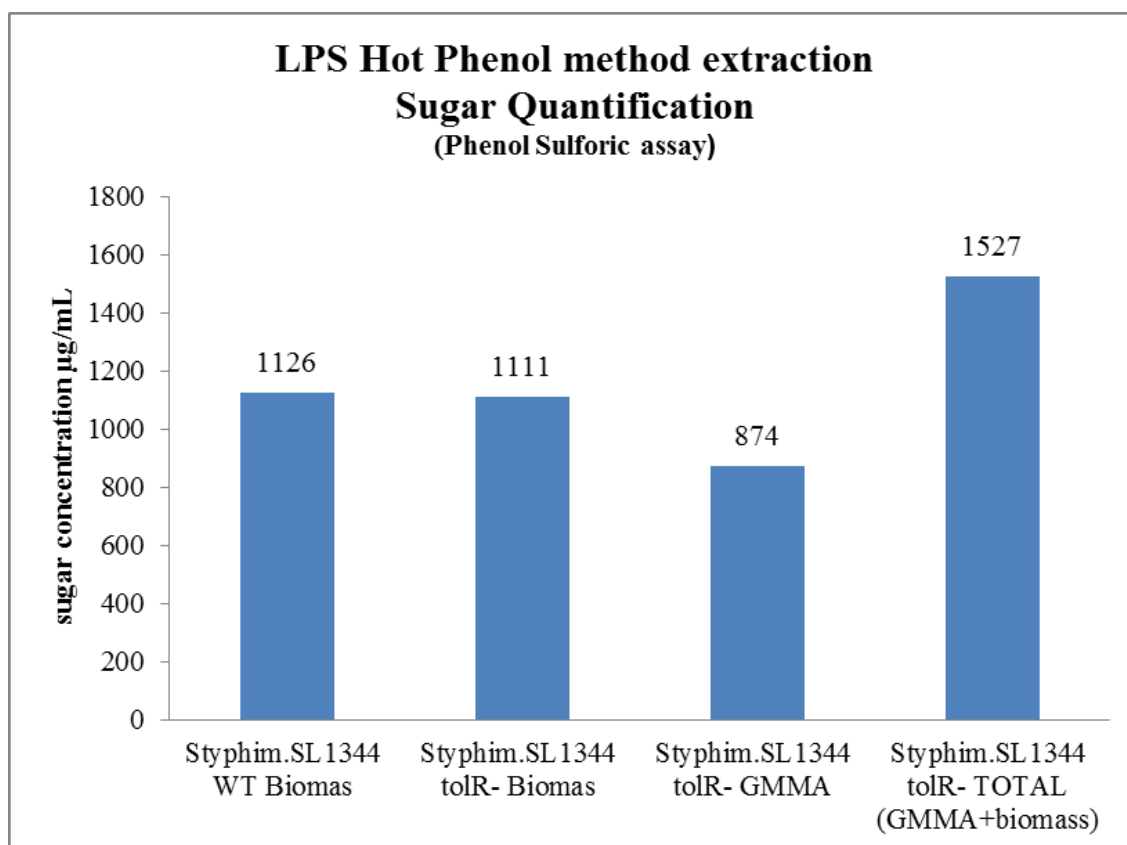


Figure 6. LPS extraction using the optimized hot phenol method for high OD samples.

GMMA from *S. Typhimurium* SL1344 *tolR*⁻ showed a comparable total sugar concentration to the *S. Typhimurium* SL1344 wild type biomass. In the *S. Typhimurium* SL1344 *tolR*⁻TOTAL sample was found the highest sugar amount, due to the presence of sugar from sugar biomass and from GMMA particles.

Total sugar quantify by the phenol sulfuric assay.

All samples were loaded on HPLC-SEC columns to visualize the O:4,5-antigen population distribution and contaminant (proteins and nucleic acids) profiles.

The Refractive index channel showed the presence of three different O:4,5-antigen s populations, based on the elution time: High Molecular Weight (HMW), Medium Molecular Weight (MMW) and Low Molecular Weight (LMW).

The *S. Typhimurium* SL1344 wild type biomass showed an enrichment in the High Molecular Weight O:4,5-antigen peak with respect to LPS from the *tolR*⁻ samples (Figure 7).

In GMMA from *S. Typhimurium* SL1344 *tolR*⁻ was found an enrichment in the Medium Molecular Weight O:4,5-antigen population (Figure 7).

S. Typhimurium SL1344 *tolR*⁻ biomass, *S. Typhimurium* SL1344 *tolR*⁻ GMMA and *S. Typhimurium* SL1344 *tolR*⁻ TOTAL (biomass+GMMA) showed a predominance of the MMW O:4,5-antigen population respect to the HMW (Figure 7).

The florescence (260/Em 280 nm) and emission (214nm) channels revealed the presence of higher amounts of impurities in LPS from the *S. Typhimurium* SL1344 wild type sample compared with LPS from GMMA from *S. Typhimurium* SL1344 *tolR*⁻ in terms of proteins and nucleic acids, indicating the purity of GMMA material respect the biomass.

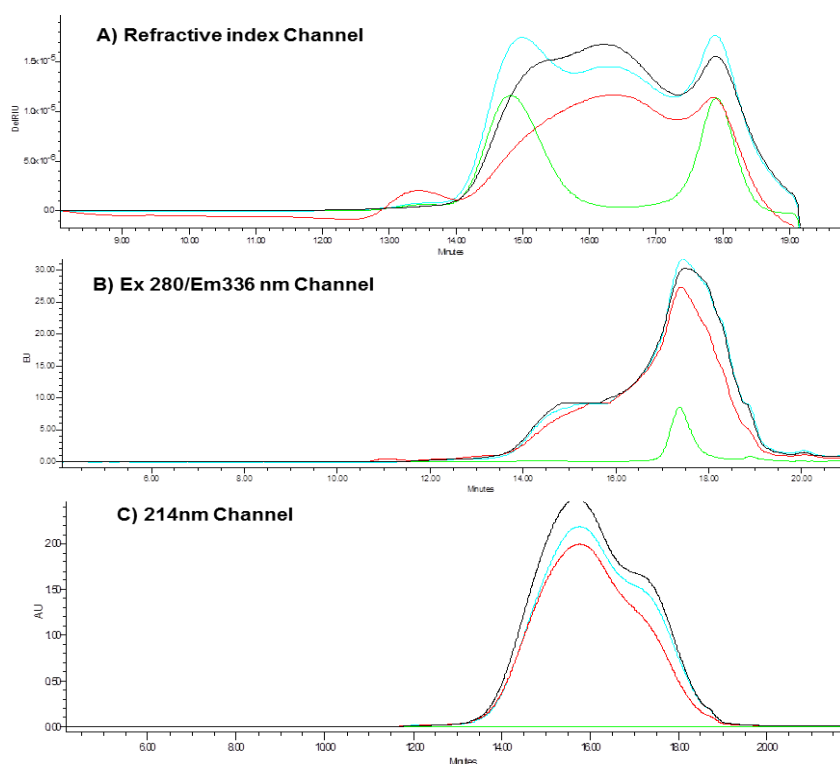


Figure 7. HPLC-SEC TSKgel 3000 PWXL column.

A) Refractive index channel: Only GMMA sample (green line), from *S. Typhimurium* SL1344 *tolR*⁻, showed two distinct O:4,5-antigen populations corresponding to the MMW and LMW. *S. Typhimurium* SL1344 Wild type sample (red line) showed an enrichment in HMW O:4,5-antigen population, corresponding with the lowest retention time peak. The GMMA sample presents an enrichment of the Medium Molecular Weight O:4,5-antigen in comparison with wild type.

All *tolR*⁻ samples showed predominance in the MMW O:4,5-antigen over the HMW population

B) Ex 260/Em 280 nm channel and **C)** 260 nm channel Refractive Index channel showed that, GMMA from *S. Typhimurium* SL1344 *tolR*⁻ sample contained less impurities than the other samples.

S. Typhimurium SL 1344 wild type: red line
S. Typhimurium SL1344 *tolR*⁻ biomass: black line
S. Typhimurium SL1344 *tolR*⁻ GMMA: green line
S. Typhimurium SL1344 *tolR*⁻ TOTAL: light blue line

These results showed that, for GMMA from *S. Typhimurium* SL1344 *tolR*⁻ there was a comparable sugar amount to *S. Typhimurium* SL1344 wild type biomass.

In GMMA particles there is a high concentration of membrane components and so GMMA could be used as a source of LPS and protein extraction and, in this case, an O4,5-antigen source.

The main difference found between *S. Typhimurium* SL1344 wild type and *S. Typhimurium* SL1344 *tolR*⁻ GMMA was related to the O:4,5-antigen populations. The GMMA sample from *S. Typhimurium* SL1344 *tolR*⁻ was enriched in MMW O:4,5-antigen with respect to the *S. Typhimurium* SL1344 wild type biomass, in which the HMW O:4,5-antigen was the most abundant population.

To verify these results and ensure reproducibility for our optimized protocol, this experiment was repeated, in parallel, 3 times (Figure 8). Samples showed a little variations in terms of total sugar concentration values but, calculating the relative coefficient of variation (Table 5), all samples showed a variability under the 15% thus our LPS hot phenol extraction protocol, optimized for high OD and GMMA, could be defined *precise* and *accurate*, in line with the FDA Guidance for Industry Bioanalytical, Method Validation (U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine (CVM), May 2001).

SAMPLE	COEFFICIENT OF VARIATION
<i>S. Typhimurium</i> SL 1344 wild type biomass	7,95
<i>S. Typhimurium</i> SL 1344 <i>tolR</i> ⁻ biomass	8,69
<i>S. Typhimurium</i> SL 1344 <i>tolR</i> ⁻ GMMA	5,24
<i>S. Typhimurium</i> SL 1344 <i>tolR</i> ⁻ TOTAL (GMMA+biomass)	2,96

Table 5. Coefficient of variation calculated on the different samples sets after the total sugar concentration estimation using the phenol sulfuric acid assay

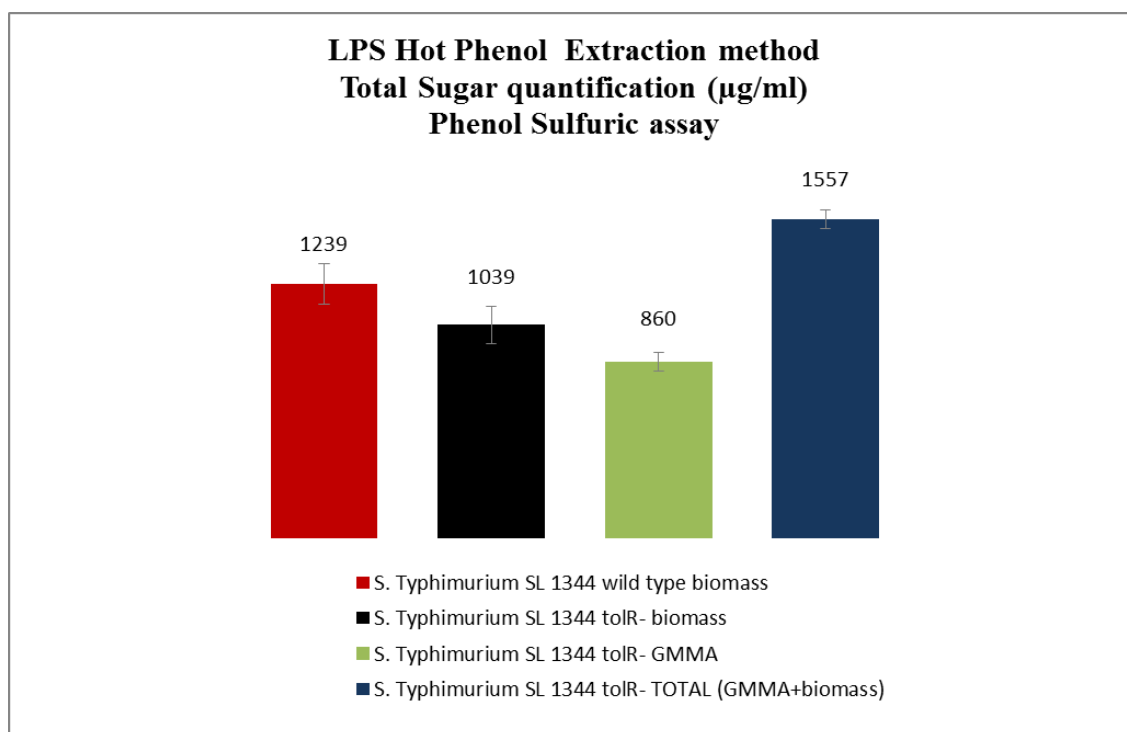


Figure 8. Consistency tests to confirm the optimization of the high OD LPS hot phenol extraction method.

Three different tests were performed individually in parallel. LPS was extracted using the optimized hot phenol extraction method.

The total sugar concentration was consistent among the different samples
 (Coefficient of variation <10%)

Total sugar concentration quantified using the phenol sulfuric assay.

3. O4:5- antigen extraction from *S. Typhimurium* SL1344 wild type, *S. Typhimurium* SL1344 *tolR*⁻ and GMMA using the Direct Acid Hydrolysis approach

In this section we describe the O:4,5-antigen extraction and purification processes using the acid hydrolysis approach, proposed by Micoli et al, applied to *Salmonella* Typhimurium SL1344 wild type biomass, *S. Typhimurium* SL1344 *tolR*⁻ and GMMA.

Samples were obtained from three fermentations performed separately but in parallel.

S. Typhimurium SL1344 Wild type was grown in a 5L fermenter using 3L of CDM to reach 30 OD, without amino acid feeding (as described in materials and method).

The direct acid hydrolysis approach was applied directly to *S. Typhimurium* SL1344 Wild type fermentation broth harvested and put in 2L glass round- bottom flask, adding 1% of Acetic Acid and boiling at 100°C for 6h. Afterwards, the sample was neutralized to pH 6-6.5. the hydrolyzed material was passed through the 0.2 TFF membrane and the permeate, containing O:4,5-antigen , was collected.

The same approach was applied for *S. Typhimurium* SL1344 *tolR*⁻ fermentation broth, in which biomass and GMMA were present. 2L of fermentation broth were harvested and hydrolyzed, using a 2L glass round- bottom flask, in acid condition at 100°C for 6h. At the end, the sample was neutralized to pH 6-6.5 and a 0.2µm TFF step was performed to collect the permeate, containing the O:4,5-antigen from biomass and GMMA material.

S. Typhimurium SL1344 *tolR*⁻ fermentation broth, from the parallel fermentation process made using another vessel, was processed using the 0.2 µm TFF membrane, to collect permeate containing GMMA. 2L of GMMA material was moved in a 2L glass round- bottom flask. 1% acetic acid was added and the sample was boiled at 100°C for 6h. At the end of the hydrolysis, this sample was centrifuged for 20 minutes, at 8000g, at 4°C (Beckman Coulter Avanti J-26 XP Centrifuge, JA-10 Fixed Angle Rotor), to divide the residual membrane fragments from the supernatant containing O:4,5-antigen .

After hydrolysis, samples were collected and analyzed in terms of total sugar content by Phenol Sulfuric Assay, peptide content by MicroBCA method and nucleic acid content by absorbance at 260 nm.

Sugar quantification showed that, GMMA from *S. Typhimurium* SL1344 *tolR*⁻ after acid hydrolysis had a comparable total sugar concentration to the hydrolyzed material from *S. Typhimurium* SL1344 Wild type (Figure 9). This was a very surprising result indicating that, the GMMA sample contains a high amount of membrane components and thus LPS and O:4,5-antigen.

The *S. Typhimurium* SL1344 *tolR* TOTAL sample showed the highest sugar concentration and this was due to the presence of biomass and GMMA from which LPS was extracted.

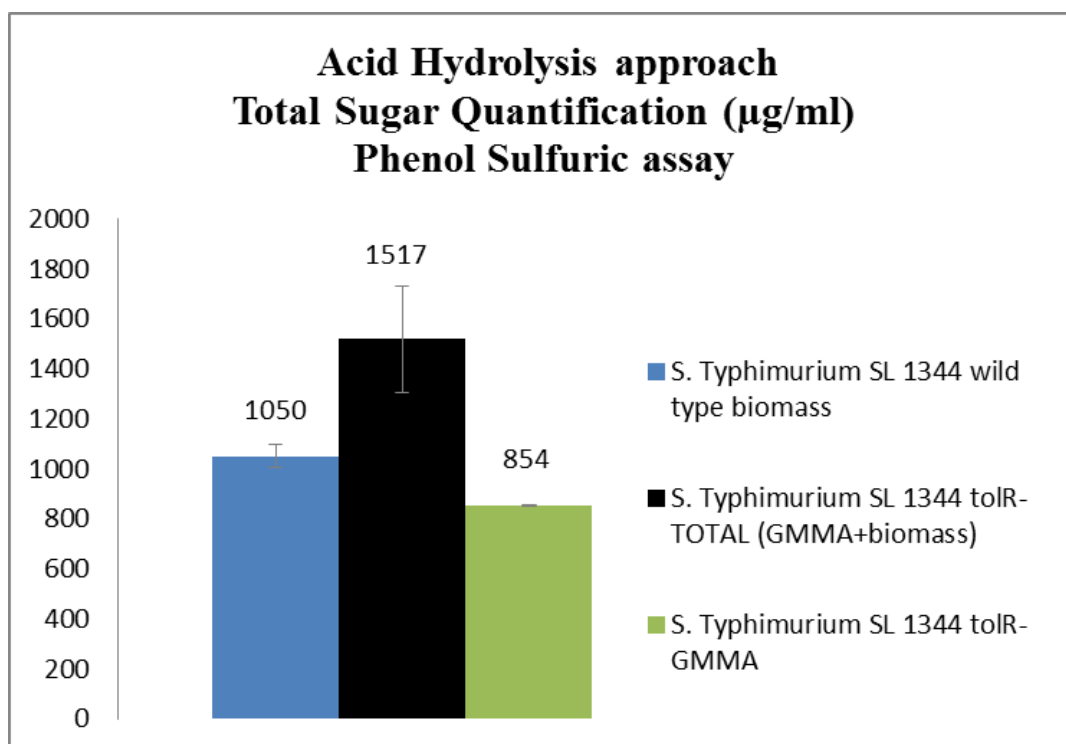


Figure 9. Direct acid hydrolysis approach to *Salmonella Typhimurium* SL1344 Wild Type *S. Typhimurium* SL1344 *tolR*⁻ total and GMMA from batch fermentation processes.

In GMMA, from *S. Typhimurium* SL1344 *tolR*⁻, was found a comparable total sugar concentration to the wild type biomass.

S. Typhimurium SL1344 *tolR* TOTAL showed the highest total sugar concentration to the other samples.

Total sugar concentration quantified by the phenol sulfuric assay.

Coefficient of variation less 10%.

All samples were characterized in terms of impurities like nucleic acid content, by absorption at 260 nm, and protein concentration, using the Micro BCA assay.

In terms of impurities, the hydrolyzed material from GMMA samples contains fewer impurities than the hydrolyzed material from *S. Typhimurium* SL1344 wild type biomass and from *S. Typhimurium* SL1344 *tolR*- TOTAL (biomass+GMMA), especially related to the nucleic acid content (Table 5).

Sample	Proteins concentration $\mu\text{g/mL}$	NucleicAcid estimation (ABS 260nm)
<i>S. Typhimurium</i> SL1344 Wild type biomass	627,1	10,08
<i>S. Typhimurium</i> SL1344 <i>tolR</i>⁻ TOTAL (biomass+GMMA)	526,4	12,71
GMMA	57,9	0,03

Table 5. Protein concentration and Nucleic Acid estimation in *S. Typhimurium* SL1344 Wild type biomass, *S. Typhimurium* SL1344 *tolR*- TOTAL (biomass+GMMA) and GMMA samples.

GMMA samples showed the lowest concentration of impurities compared with *S. Typhimurium* SL1344 Wild type biomass and *S. Typhimurium* SL1344 *tolR*- TOTAL, especially related to Nucleic acid content.

These samples were loaded on HPLC-SEC columns to determine the O:4,5-antigen profile. The Refractive Index detector revealed the presence of three different O:4,5-antigen populations.

The hydrolyzed material from *S. Typhimurium* SL1344 wild type showed a main peak corresponding to the High Molecular Weight O:4,5-antigen (HMW, Figure 10) and another one corresponding to Medium Molecular Weight O:4,5-antigen (MMW).

The hydrolyzed material from GMMA showed a main peak corresponding to Medium Molecular Weight O:4,5-antigen (MMW) and another one for the Low Molecular Weight O:4,5-antigen (LMW) (Figure 10).

S. Typhimurium SL1344 *tolR*⁻ TOTAL (biomass+GMMA) showed the same O:4,5-antigen population found in GMMA (Figure 10).

The HPLC-SEC profile showed that, the main difference between *S. Typhimurium* SL1344 wild type and *S. Typhimurium* SL1344 *tolR*⁻ samples, GMMA and TOTAL, was related to the O:4,5-antigen population.

In particular, *S. Typhimurium* SL1344 wild type biomass sample showed the predominance of the HMW O:4,5-antigen population while in GMMA, from *S. Typhimurium* SL1344 *tolR*⁻, the main peaks were related to the MMW and LMW O:4,5-antigen (Figure 10).

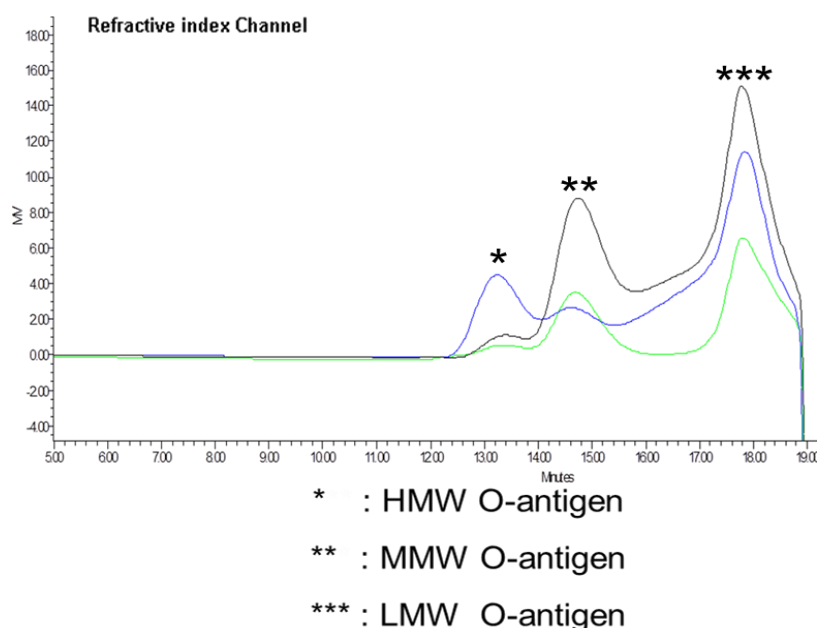


Figure 10. HPLC-SEC TSKgel 3000 PWXL column, Refractive index channel.

Wild type sample (blue line) showed an enrichment of HMW O:4,5-antigen population, corresponding to the lowest retention time peak, respect the MMW population. All samples derived from *S. Typhimurium* SL1344 *tolR*⁻, GMMA (green line) and TOTAL (black line), showed predominance in the MMW O:4,5-antigen respect the HMW population.

4. O:4,5-antigen purification process from *S. Typhimurium* SL1344 wild type and *S. Typhimurium* SL1344 *tolR*⁻ GMMA

A. O:4,5-antigen purification from *S. Typhimurium* SL1344 wild type

O:4,5-antigen from biomass of *S. Typhimurium* SL1344 wild type was purified, after acid hydrolysis, as described by Micoli et al.

The standard purification protocol, developed for O:4,5-antigen recovery from the biomass at the end of the fermentation process, consists in several steps necessary to remove impurities. First step is the 30KDa Tangential Flow Ultrafiltration. The resulting retentate is treated using 200 mM citrate buffer at pH 3, which allows the co-precipitation of nucleic acids and proteins. This material is also passed through a cationic chromatographic filter (Sartobind S MA75), in order bind residual proteins. The resulting flow through is then treated with CaCl/EtOH buffer to promote the precipitation of the remaining impurities, especially nucleic acids. At the end, the purified O:4,5-antigen contains less than 1% of proteins and nucleic acids [27,158,159].

<i>S. Typhimurium</i> SL1344 Wild type	Sugar (ug/mL)	Protein (ug/mL)	Nucleic acid (ug/mL)	% Protein	% Nucleic acid
first 30 KDa TFF	4580	822,6	2850	18	62,2
first citrate buffer precipitation	4692	90	800	1,9	17
Sartobind S MA75	4135	16,2	15,1	0,4	0,4
second CaCl/EtOH precipitation	3250	6	13,2	0,2	0,4

Table 6. O:4,5-antigen Purification Process from biomass of *S. Typhimurium* SL1344 Wild type.

Purified O:4,5-antigen contains less than 1% of impurities.

B. Comparison of four different O: 4,5-antigen purification processes starting from GMMA produced by *S. Typhimurium* SL1344 *tolR*

In this section we describe the O:4,5-antigen purification process, already proposed by Micoli et al, applied to purification of O-antigen from GMMA.

We want to explore the possibility of using GMMA as an alternative O:4,5-antigen source with respect the conventional bacterial biomass.

In order to optimize the O:4,5-antigen purification protocol from GMMA, we tested different purification conditions to increase the recovery in terms of sugar yield and to decrease processing time and relative costs.

GMMA sample showed a starting sugar concentration (859 µg/mL) comparable with the *S. Typhimurium* SL 1344 wild type biomass.

Even at the beginning of the purification protocol, GMMA showed a very low amount of impurities, especially in relation to nucleic acids (Table 7), which at the starting point were already less than 1%.

Following step by step the standard O:4,5-antigen purification process applied to GMMA samples we noted that, at the end of the precipitation steps, no pellets were found. This was probably due to the absence of enough proteins and nucleic acids necessary for the precipitation of visible pellets.

Based on the nucleic acids estimation, very low levels of this contaminant were present in GMMA samples. Thus after the first 30KDa TFF and after the first precipitation step with citrate buffer, the amount remains approximately the same (Table7).

<i>S. Typhimurium</i> SL1344 - <i>tolR</i>	Sugar	Protein	Nucleic acids	%	%
GMMA	(ug /mL)	(ug/mL)	(ug/mL)	Protein	Nucleicacid
first 30 KDa TFF	2471,8	211,6	9,05	8,6	0,4
first citrate buffer precipitation	2438,5	122,2	6,15	5	0,3
Sartobind S MA75	2060	8,1	5,46	0,4	0,3
second CaCl/EtOH precipitation	1542,04	6,8	4	0,4	0,3

Table 7. Standard purification process applied to GMMA.

Sugar concentration was quantified by phenol sulfuric assay, residual peptide by micro-BCA and nucleic acid by A260. Percentage calculation related to the total sugar amount in the specific volume obtained during each step of the purification protocol

A different scenario was found for residual proteins, where their content decreased drastically, by about 50%, at the end of the first precipitation step (Table 2) and another important reduction was found after the cationic exchange chromatography.

Based on these results, we may argue that it could be possible to remove some purification steps from the standard original protocol.

In order to confirm this hypothesis and to understand the better procedures in order to purify O:4,5-antigen from GMMA, different purification strategies were applied to the retentate material, post 30KDa TFF (Figure 11).

We compared the final sugar concentration, obtained from the standard purification protocol applied on GMMA, with different combinations of the purification steps:

- Only precipitation using the citrate buffer
- Precipitation with citrate buffer in combination with cationic exchange chromatography
- First precipitation step with citrate buffer followed directly by precipitation with CaCl₂/EtOH
- Only the cationic exchange chromatography, using the Sartobins S MA75 filter (Figure 11).

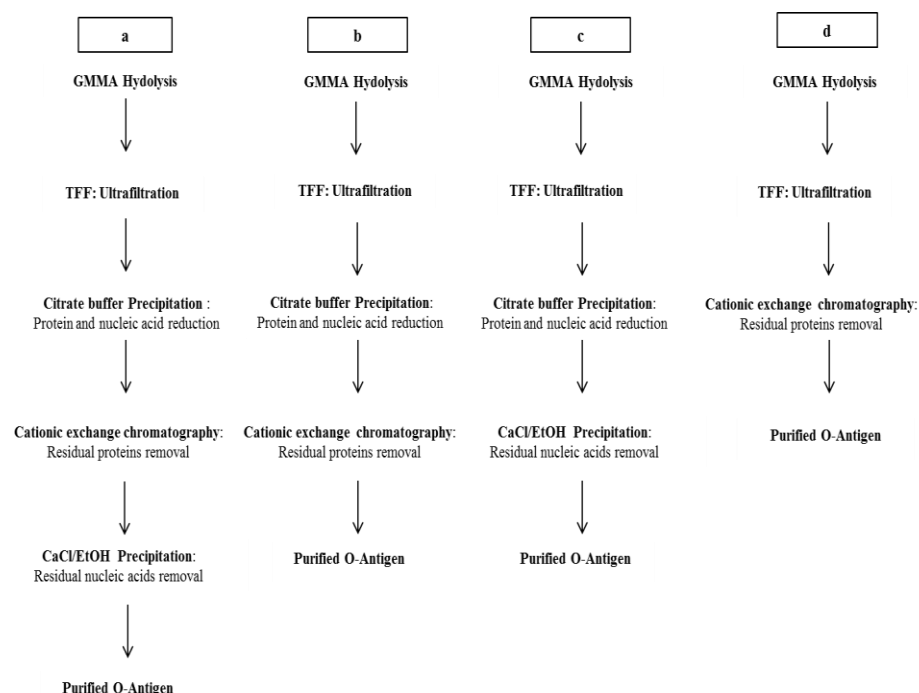


Figure 11. O:4,5-antigen purification strategies starting from GMMA.

Combinations of purification steps: a) GMMA sample was treated using the standard O:4,5-antigen purification protocol, already developed for the biomass; b) Only the first precipitation step and the cationic exchange chromatography were used; c) Citrate buffer and CaCl/EtOH precipitations were tried avoiding the Sartobind S; d) Only the cationic exchange chromatography was used

The first precipitation step using the citrate buffer 20mM, was not sufficient to reduce significantly the residual proteins amount, therefore further optimization to remove impurities are necessary.

A similar result was found using only the two precipitation steps (citrate buffer and CaCl/EtOH), avoiding the Sartobind S MA75 filter step. Also here the residual proteins and nucleic acid level remained high.

The purification process worked better using the first precipitation step with citrate buffer 20mM in combination with the cationic exchange chromatography. At the end of these two steps, the residual nucleic acid and proteins decreased dramatically and also a good sugar amount was obtained (Table 8).

Based on these results we tried only the Sartobind S MA75 filter, without the precipitation step. The retentate of 30KDa TFF after hydrolysis of GMMA was buffered with 20mM citrate and directly passed through the Sartobind S chromatographic filter, previously conditioned with the same buffer. A consistent decrease in terms of impurities was found. The total sugar amount in the flow through after cationic exchange chromatography was higher than the final sugar concentration in the standard purification protocol (Table 8).

<i>S. Typhimurium</i> SL1344 -tolR GMMA	Sugar (ug/mL)	Protein (ug/mL)	Nucleic acid (ug/mL)	% Protein	% Nucleic Acid
Classic purification process	1542	6,8	4	0,4	0,3
First citrate buffer precipitation + second CaCl/EtOH precipitation	1692	66,2	5,6	3,9	0,3
Only cationic exchange chromatography	1772	5,1	3,3	0,3	0,2

Table 8. Final sugar concentration comparison between the standard purification process and different purification step combinations.

Sugars concentration was quantified by phenol sulfuric assay, residual peptide by micro-BCA and nucleic acid by A260. Percentage calculation related to the total sugar amount in the specific volume obtained during each step of the purification protocol

Starting from GMMA, the purification protocol could be drastically reduced. Cationic exchange chromatography, previously conditioned with the citrate buffer, could be used as the only step necessary to remove all impurities and could be applied directly to the retentate after 30KDa TFF.

5. O4:5- antigen from *Salmonella* Typhimurium SL1344 *tolR*⁻ GMMA: characterization of the Medium Molecular Weigh population

In order to ensure the equivalence of the O antigen from GMMA with the O antigen from *S. Typhimurium* SL1344 wild type [162,163], a comparison in terms of sugar composition and structure was performed.

We have focused our attention on the MMW O:4,5-antigen extracted from *S. Typhimurium* SL1344 *tolR*⁻ GMMA.

Starting from GMMA, at the end of the standard purification protocol, a significant sugar decrease was found, due to the loss of the LMW O:4,5-antigen population, as show in the HPLC-SEC profiles in Figure 12, leaving MMW O:4,5-antigen as the predominant population.

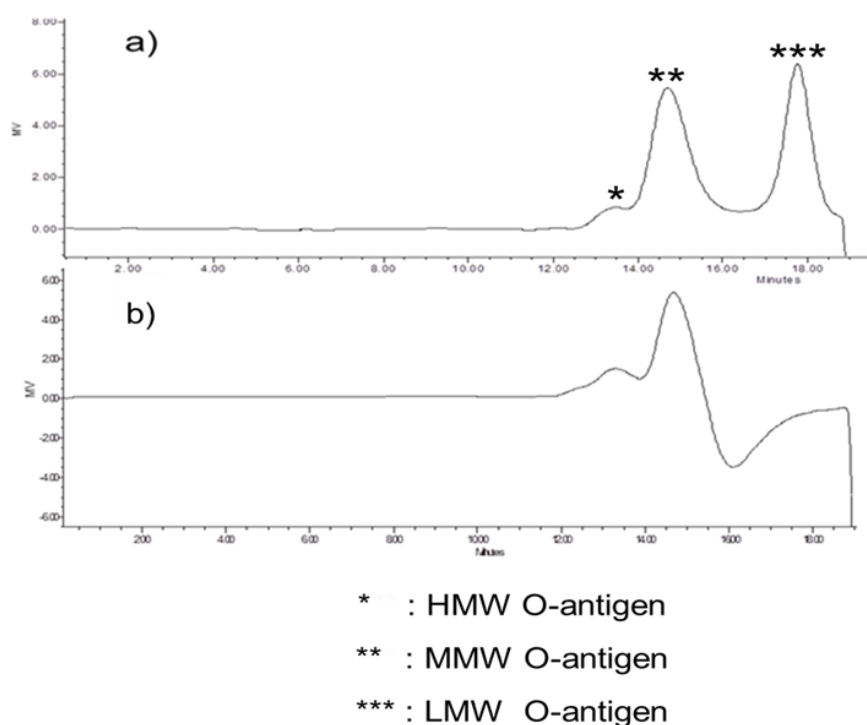


Figure 12. O 4,5-Antigen GMMA profiles

- a) O 4,5-antigen profile in GMMA sample before the purification process, three O:4,5-antigen populations were present, HMW, MMW and LMW
- b) O 4,5-antigen profile in GMMA sample, after the first 30KDa tangential flow ultrafiltration step the LMW O:4,5-antigen population was completely lost

The O:4,5-antigen mix, at the end of the purification protocol from GMMA, was loaded on Sephacryl S-100 HR 16/60 Gel Filtration column to separate the HMW population, present as a minimal portion, from the MMW O:4,5-antigen.

Fractions corresponding to the MMW O:4,5-antigen were collected, pooled and further characterized.

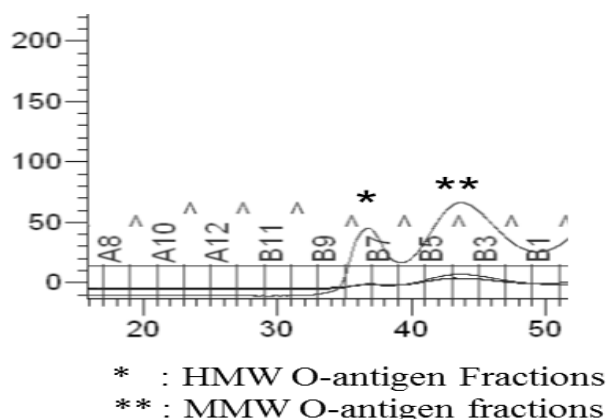


Figure 13. O 4,5-Antigen GMMA profiles loaded on Sephacryl S-100 HR 16/60 Gel Filtration column.

Purified O 4,5-antigen from GMMA was loaded on S-100 and the fractions corresponding to the HMW and MMW O:4,5-antigen were collected and pooled

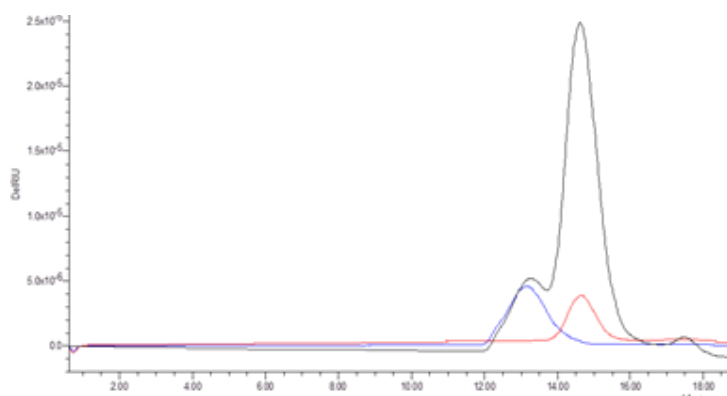


Figure 14. MMW and HMW O 4,5-Antigen GMMA profiles loaded on HPLC-SEC (Refractive index detector) TSKgel 3000 PWXL column.

Comparison among the purified O 4,5-antigen from GMMA (black line), the HMW (blue line) and MMW (red line) pooled fractions from **Sephacryl S-100** column. This was a

confirmation of that, the corresponding fractions of the HMW and MMW O:4,5-antigen s were correctly pooled from the Sephacryl S-100 size exclusion chromatography.

The pool containing the MMW O:4,5-antigen from GMMA of *S. Typhimurium* SL1344 *tolR*⁻ was analyzed by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD), to understand the structure and the similarities with *S. Typhimurium* SL1344 wild type.

MMW O:4,5-antigen from GMMA was found to contain galactose, rhamnose, mannose at a ratio of 1:1 but with a decreased level of glucosylation (relative to rhamnose).

As expected, the chain composition of MMW O:4,5-antigen from GMMA of *S. Typhimurium* SL1344 *tolR*⁻ was found to be the same found in the wild type strain [162,163] (Table 9).

Sugar	<i>S. Typhimurium</i> SL1344 <i>tolR</i> ⁻ GMMA*
Rha	1
Man	1
Gal	1,08
Glc	0,49

*: molar ratios relative to Rha

Table 9: Sugar composition of Medium Molecular Weight O:4,5-antigen from *S. Typhimurium* SL1344 *tolR*⁻ GMMA. Commercial standard for Abe is not available

6. *S. Typhimurium* SL1344 *tolR*⁻ GMMA: recovery process optimization

NVGH has developed a new method for GMMA recovery at the end of the fermentation process with a tangential flow filtration step using a 0.2 µm membrane to remove bacteria from the culture supernatant, and a second tangential flow filtration step, with a 0.1 µm membrane, to concentrate GMMA and to remove soluble proteins [27,129,158,159].

In this paragraph, we describe the optimization of GMMA recovery process based on the TFF membrane cut off screening applicable on GMMA produced by *S. Typhimurium* SL1344 *tolR*⁻. In the published literature it is already reported that, *Salmonella* Typhimurium wild type produces GMMA with size in a range of 10 to 200 nm [164]. Thus, based on this information, to find the best condition for recovery of all GMMA after the first 0.2 µm TFF, we tested different TFF cut off membranes.

S. Typhimurium SL1344 *tolR*⁻ was fermented as previously described. GMMA were collected at the end of the fermentation process using the 0.2 µm TFF membrane. 1L of the resulting permeate was processed using sequential TFF steps decreasing the membrane cut off: 0.1 µm → 500KDa → 100KDa → 30KDa.

To obtain more concentrate samples, 20mL from all retentate (after 0.1µm, 500KDa, 100KDa and 30KDa) and only the 30 KDa permeate were ultracentrifuged at 180 000g, 2h at 4°C. The resulting pellets were resuspended in 5 ml of PBS and further sterilized with a 0.2 µm filter (Millipore).

Interesting results were found in the retentate and permeate from the 30KDa TFF step: no pellets were found in both samples after ultracentrifugation. The absence of GMMA pellets in the 30KDa retentate and permeate indicated that GMMA were not present in these samples. Therefore the 100KDa cut off membrane was able to retain GMMA.

To extract O:4,5-antigen from each sample and to quantify the total sugar amount, 2mL from all samples resuspended in PBS was put in glass vials and treated with the direct acid hydrolysis approach (1% acetic acid, 100°C, 6h)

The phenol sulfuric quantification showed that, GMMA in the 100KDa retentate had the highest total sugar concentration (516.53 µg/mL) compared with the other samples. In contrast, GMMA in the 0.1µm retentate showed the lowest (51.56 µg/mL) total sugar amount (Figure 15).

These results suggest that some GMMA passed through both the 0.1 μm and 500KDa TFF membranes, probably this was due to a not uniform GMMA size distribution in the samples. The bigger GMMA were retained by the 0.1 μm cassette, and particles with an intermediate size range were collected by the 500KDa membrane. Thus only the 100KDa TFF membrane was able to retain small sizes GMMA (for more details see the electron microscopy section). The comparison between the total sugar concentration found in the 0.2 μm permeate, containing all sizes of GMMA (851 $\mu\text{g/mL}$), and the sum of the total sugar amounts from each retentate were comparable (GMMA in the 100KDa retentate 516.53 $\mu\text{g/mL}$ + GMMA in the 500KDa retentate 187.11 $\mu\text{g/mL}$ + GMMA in the 0.1 μm retentate 51.56 $\mu\text{g/mL}$ = 755 $\mu\text{g/mL}$ total), so during the screening process more than the 90% of produced GMMA were recovered (Figure 15).

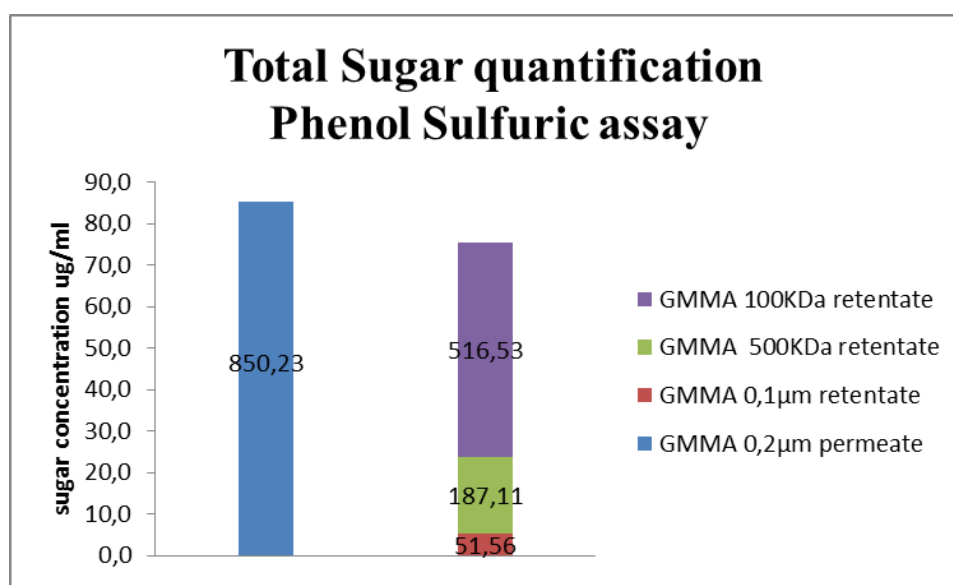


Figure 15. GMMA after the 0.2 μm , 0.1 μm , 500KDa and 100KDa TFF steps.

Total Sugar concentration.

The highest sugar concentration was found in the 100KDa retentate (purple). The 0.1 μm (red) and 500KDa (green) membranes were able to collect GMMA with a bigger diameter so the smallest particles were retained by the 100KDa membrane. In the GMMA 0.2 μm permeate (blue) all GMMA, with different sizes, were present. The total GMMA sugar quantity was similar to the sum of that present in the different retentates, indicating that, during the TFF process, 94% of produced GMMA were recovered.

We have demonstrated that, *S. Typhimurium* SL1344 *tolR*⁻ produces GMMA with different size and this is a crucial factor for the GMMA recovery process. The membrane cut off must be chosen for each strain based on the specific GMMA size, in order to collect all material maximizing the yield.

The best choice in order to recover all GMMA produced by *S. Typhimurium* SL1344 *tolR*⁻, after the first 0.2 μm TFF step, is the 100KDa membrane. This was confirmed by the total sugar concentration found after the GMMA acid hydrolysis, at the end of this screening.

7. ELECTRON MICROSCOPY RESULTS

At the end of fermentation process, 50 mL of *S. Typhimurium* SL1344 *tolR*⁻ bacterial culture were centrifuged to divide the biomass from the supernatant. The resulting biomass pellet was directly prepared and fixed to be visualized by Transmission Electron Microscopy. The supernatant was ultracentrifuged and the resulting pellet was resuspended in PBS to obtain the sample called as “*GMMA before the 0.2 um TFF*”, further treated for TEM visualization.

As show in Figure 16, *S. Typhimurium* wild type strain shows a thick and straight outer membrane layer in direct contact with the periplasm. The OM, the periplasm and the IM form together a compact and well organized structure [165]. On the contrary, the *S. Typhimurium* SL1344 *tolR*⁻ bacterial cell showed an instable OM layer and an abundant periplasmic interlayer space. This interspace, between the OM and the IM, is not well organized as in the wild type strain. This was the consequence of the *tolR* gene deletion, which encode for a protein parts of the Tol-Pal system responsible to maintain the integrity of the outer membrane.

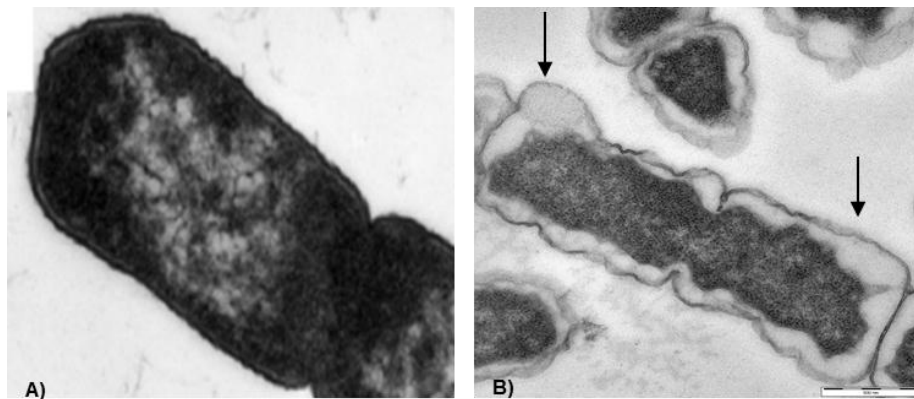


Figure 16. *S. Typhimurium* wild type strain and *S. Typhimurium* SL1344 *tolR*⁻ bacterial cells.

A) *S. Typhimurium* wild type bacterial cell shows a well-organized OM-periplasm-IM structure

B) *S. Typhimurium* SL1344 *tolR*⁻ shows a compromised cell envelope structure.

Arrows indicates the membrane instability portions

(ref. bar is 500 nm)

At the end of the *S. Typhimurium* SL1344 *tolR*⁻ fermentation process 100 µL of fermentation broth, composed by bacterial cells and GMMA, were collected and directly prepared for TEM analysis.

We captured the nascent formation of GMMA on *S. Typhimurium* SL1344 *tolR*⁻ bacterial cells (Figure 17), showing the presence of these particles in different location on the bacteria surface.

GAMMA releases occur along cell body and also in correspondence of the division septa (Figure 18), this phenomenon was already observed by Deatherage BL et al. in *S. Typhimurium* *tolA*⁻ mutant strains [166].

In Gram negative bacteria, the defective linkage between the Outer Membrane and the Inner Membrane influences the GMMA releases and, probably, this defective linkage modulates the GMMA production at the division septa [167] as shown in Figure 18.

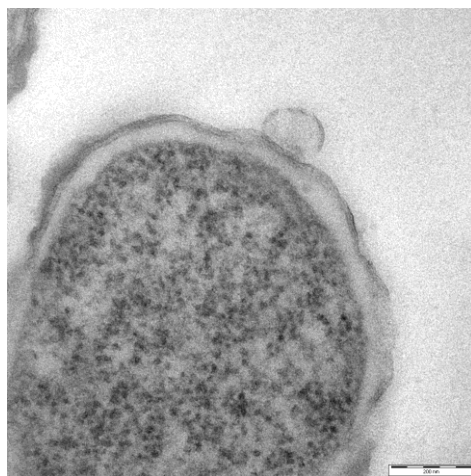


Figure 17. Nascent GMMA from *S. Typhimurium* SL1344 *tolR*⁻ bacteria along the cell body (ref. bar is 200nm).

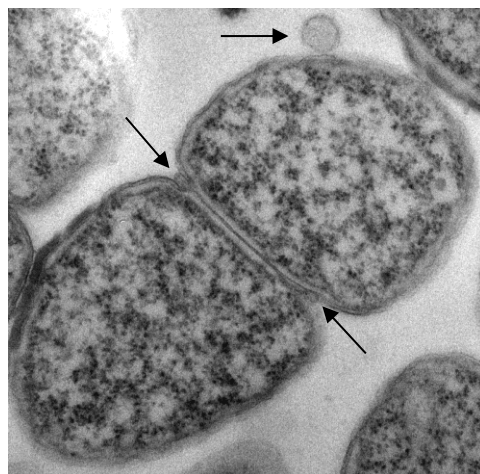


Figure 18. GMMA release from *S. Typhimurium* SL1344 *tolR*⁻ along cell body and at division septa. Arrows indicate the nascent GMMA from division septa and along the cell body. (ref. bar is 200nm)

The fermentation broth taken directly from the vessel, before any treatment and directly visualized by TEM microscope showed a complex preparation composed of a mixture of bacteria, GMMA and a relevant amount flagella, as shown in Figure 19. However, also in sample “*GMMA before the 0.2 um TFF*” (Figure 5), obtained after ultracentrifugation, a massive presence of flagella was observed. These results suggest that, during the growth of *S. Typhimurium* SL1344 *tolR*⁻, flagella are naturally released in the fermentation broth.

The observation of “*GMMA before the 0.2 um TFF*” sample has shown a non-homogeneous composition not only due the presence of flagella but also due the massive presence of GMMA particles with a wide range in size distribution, as shown in Figure 20.

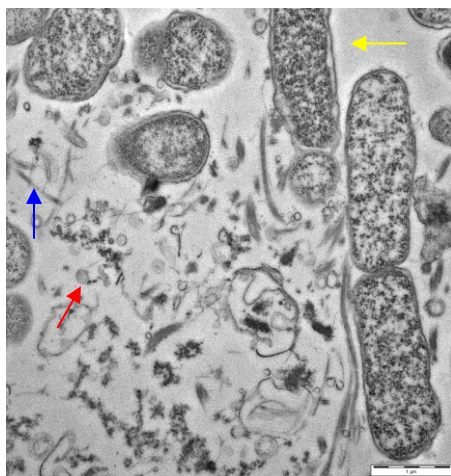


Figure 19. fermentation broth of *S. Typhimurium* SL1344 *tolR*⁻.
Bacterial cell (yellow arrow), GMMA (red arrow) and flagella (blue arrow).

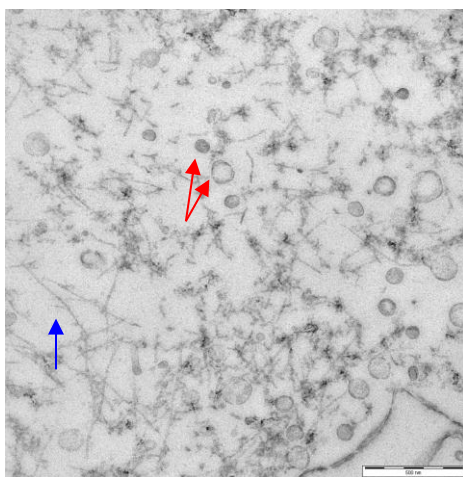


Figure 20. GMMA before the 0.2 μ m TFF sample.
Flagella (blue arrow) and GMMA particles (red arrows) with different size

A. Negative Staining

The negative staining approach was applied on all the retentate samples collected during the TFF cassettes Pore size screening for GMMA recovery, as described in materials and methods section.

The “GMMA 0.1 μm retentate” sample (collected after the 0.1 μm pore size filter cassette TFF) showed a huge amount of flagella and also particles with an average size distribution of 56 nm (size range: 25-132nm). GMMA and flagella, stick to each other, formed a sort of “grid structure” able to entrap GMMA particles, as shown in Figure 19.

This interaction might affect the free passage of GMMA particles through the 0.2 μm and 0.1 μm TFF membranes reducing the efficacy of the GMMA recovery thus the related processes.

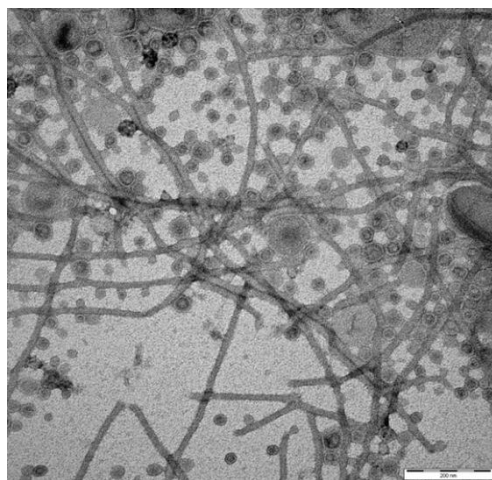


Figure 19. GMMA 0.1 μm retentate sample (ref. bar is 200nm)

The scenario was quite different in the “GMMA 500KDa retentate” and “GMMA 100KDa Retentate” samples, collected after the 500KDa and 100KDa pore size filter cassette TFF steps. Looking at these samples, the presence of flagella, in both samples, decreased dramatically respect to the “GMMA 0.1 μm retentate” thus almost all flagella were retained during the 0.1 μm TFF step while the majority of GMMA passed through this cut-off membranes, confirming the suitable use of the 0.1 μm cut-off TFF as a good and rapid way to collect flagella.

The negative staining of the “*GMMA 500KDa retentate*” samples showed a particles size average of 36 nm, in a range from 20 nm to 97 nm (Figure 20).

In “*GMMA 100KDa retentate*” sample the particles average size was 30 nm, with a range from 17 nm to 48nm (Figure 21).

Based on these results we can say that, *S. Typhimurium* SL1344 *tolR*⁻ strain produces GMMA with different size, in a range from 130 nm to 17 nm.

The 100 KDa membrane cassette was able to retain quantitatively all different GMMA particles produced by *S. Typhimurium* SL1344 *tolR*⁻ strain, which passed, with a different ratio, through the 0.1µm and 500KDa cut-off membranes. This data confirms the results shown before, obtained by the total sugars quantification after the GMMA direct acid hydrolysis in the TFF cassettes Pore size screening for GMMA recovery section.

In conclusion, *S. Typhimurium* SL1344 *tolR*⁻ release preferentially small GMMA and the best TFF cassette to recover all particles, at the end of the fermentation process and after the first 0.2 µm TFF step, is the 100 KDa cut-off membrane filter for tangential flow ultrafiltration.

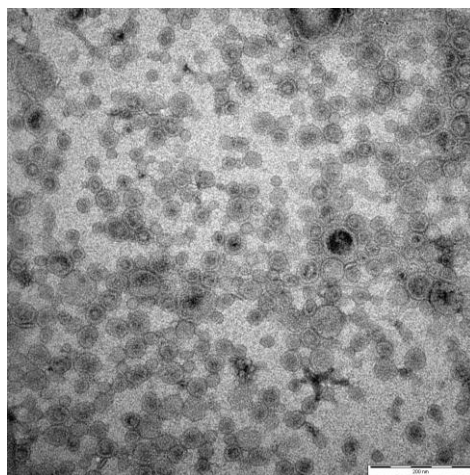


Figure 20. *GMMA 500KDa retentate* sample (ref. bar is 200nm)

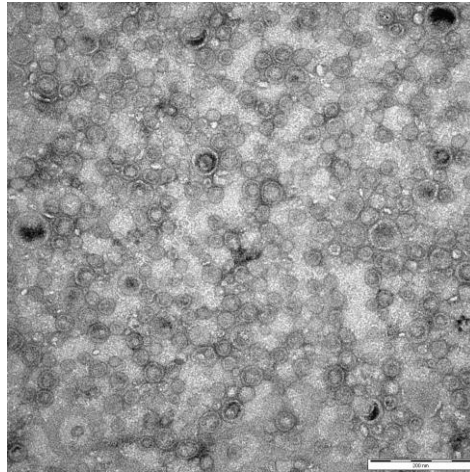


Figure 21. GMMA 100KDa retentate sample (ref. bar is 200nm)

B. Transmission Electron Microscopy

The observation using Transmission Electron Microscopy (TEM) was applied on “GMMA 0.1 retentate”, “GMMA 500KDa retentate” and “GMMA 100KDa retentate” samples.

Analyzing pictures from each sample, a curious structure was shown: small GMMA particles were found to be encapsulated in the inner space of a bigger one GMMA (Figure 22).

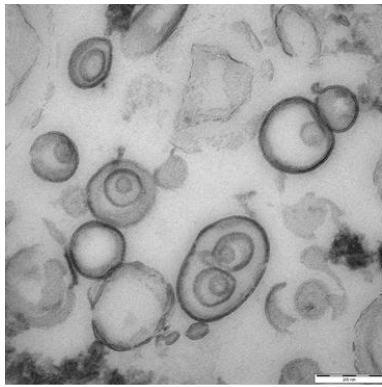
The same scenario was found in each sample indicating that, GMMA forms a complex structure, interacting each other, and the smallest particles were encapsulated inside of a bigger GMMA.

The origin of the mechanism, which leads this phenomenon, and how GMMA particles interact to each other to form this structure, are still unknown thus further investigation are required.

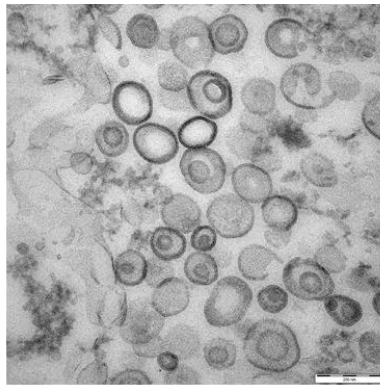
We also noted that, some GMMA particles born from the same outer membrane point and at this stage the single particles could fuse each other, interacting in an unknown manner, and making the “GEMMA inside GMMA” structure (Figure 23).

Another plausible explanation could be related to the release of a big GMMA able to lead away other outer membrane portions, which remain in its internal space, forming particles (Figure 24).

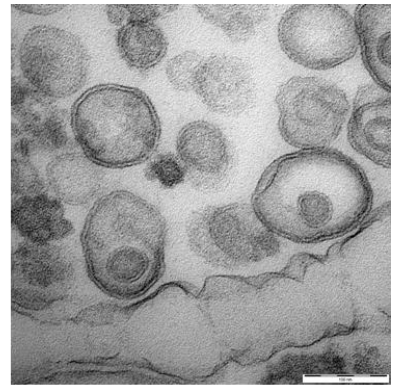
The explanation of the encapsulation phenomenon remains still unknown and further investigations are required.



A) GMMA 0.1 μ m retentate



B) GMMA 500KDa retentate



C) GMMA 100 KDa retentate

Figure 22. The “GMMA inside GMMA” phenomenon.

(in A and B ref. bar is 200nm; in C ref. bar is 100nm)

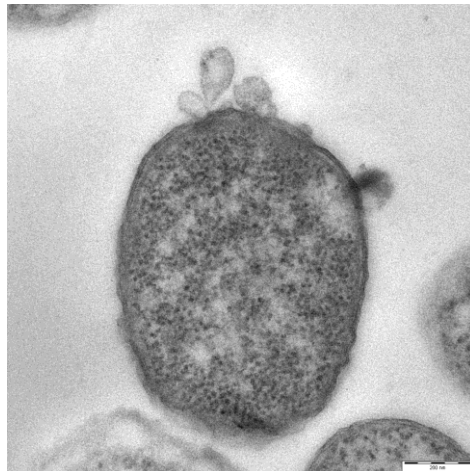


Figure 23. GMMA particles from the same membrane point

(ref. bar is 200nm)

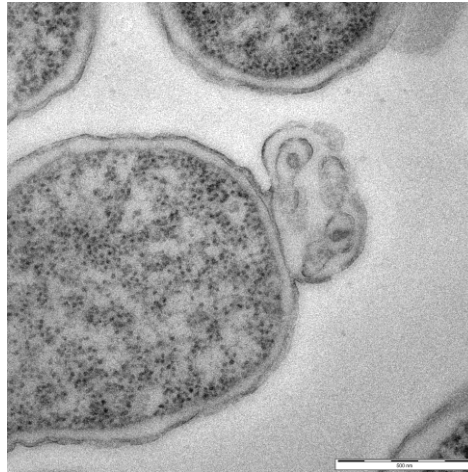


Figure 24. Small GMMA encapsulated in the internal space of a bigger one GMMA.
(ref. bar is 500nm)

VI. GENERAL DISCUSSION

1. Summary of Results

In nature, Gram-negative bacteria normally shed parts of their outer membrane via membrane particles, these are usually known in literature as outer membrane vesicles. In NVGH, we have named these membrane particles as GMMA (Generalized Modules for Membrane Antigens) to distinguish them to the detergent extracted OMVs, which differ from native GMMA in composition and immunogenicity [168].

GMMA composition is representative of the bacterial outer membrane, their surface is composed of outer membrane proteins, lipopolysaccharide (LPS), phospholipids and soluble periplasmic proteins.

The porpoise of my work was to demonstrate the possibility to use GMMA as a new source of immunogenic membrane components, like proteins and LPS, useful for vaccines development. I have chosen, as target membrane molecules, the LPS and in particular the O:4.5 antigens polysaccharide, extracted from GMMA produced by *S. Tyhphimurium* SL1344 *tolR*⁻ strain, in

which the knockout of *tolR* gene, promotes GMMA release by decreasing the outer membrane stability.

The first step was to maximize the productivity of GMMA during the fermentation process, as well as their recovery from the fermentation broth.

A chemically defined medium was formulated bespoke and optimized to create the best grow conditions for *S. Tyhphimurium* SL1344 *tolR*⁻ strain. Different conditions were tested and, at the end, this bacterial strain showed an auxotrophy for histidine thus without this amino acid *S. Tyhphimurium* SL1344 *tolR*⁻ is not able to grow.

S. Tyhphimurium SL1344 *tolR*⁻ was fermented in a 5L fermenter with 3L of working volume up to 30 OD and, after the oxygen spike, the harvest was performed.

In parallel, the wild type strain was fermented, in the same conditions, without histidine feedings, up to 30 OD.

NVGH has developed a new purification method to recover GMMA produced by different *tolR*⁻ Gram negative strains, using two consecutive TFF steps. In the first step, the 0.2 µm cut-off membrane is used to separate the biomass, which remains in the retentate, from the GMMA particles that pass through the filter cassette in the permeate.

The permeate is then further passed through another TFF filter membrane with 0.1 µm cut-off, necessary to separate GMMA particles, which remains in the retentate, from soluble proteins that pass through the filter cassette in the permeate.

GMMA from *S. Tyhphimurium* SL1344 *tolR*⁻ were collected using the new method developed in NVGH but the process was further optimized in order to maximize the GMMA yield.

To optimize the GMMA recovery process the key factor is the second TFF step. The membrane cut-off must be chosen respect GMMA sizes [165] and must be able to retain, as much as possible, GMMA particles.

We tested different cut-off membranes (0.1 µm, 500 KDa and 100 KDa) in order to know the best one able to retain all GMMA particles produced by *S. Tyhphimurium* SL1344 *tolR*⁻. Satisfactory result was obtained using the 100 KDa cut-off membrane, with a GMMA particles retention more than 90%.

In conclusion, GMMA particles could be produced, from *S. Tyhphimurium* SL1344 *tolR*⁻, during batch fermentation process in CDM supplemented with histidine, and the GMMA recovery process could be simple and fast using only two consecutives TFF steps.

To characterize the O:4,5-antigen of *S. Tyhphimurium* SL1344 *tolR*⁻ GMMA and also to have a comparison with the *S. Tyhphimurium* SL1344 wild type polysaccharide, LPS was extracted from GMMA and from the wild type biomass using the hot phenol method, optimized for high OD bacterial culture and for GMMA samples. The O-antigen was cleaved from the extracted LPS using the acid hydrolysis approach. The total sugars quantification, in the hydrolyzed samples, was done using the phenol sulfuric assay. The total sugar concentration, after extraction and hydrolysis, in the *S. Tyhphimurium* SL1344 wild type biomass was comparable with the total sugar concentration observed in GMMA, obtained from *S. Tyhphimurium* SL1344 *tolR*⁻.

The hot phenol extraction method is known to be the best choice for the LPS recovery but it can be applied only for a small samples volume due the severe toxicity of phenol, not applicable to industrial process.

Recently, Micoli et al. have proposed an alternative method for the O-antigen extraction method directly from the biomass, avoiding the phenol use [165,169]. The method consists essentially in a direct acid hydrolysis of the bacterial culture, at the end of fermentation process, for 6 hours at 100°C. In these conditions, the O-antigen is cleaved from the Lipid A and released in the fermentation broth. These conditions were tested on GMMA sample to extract O:4,5 -antigen polysaccharides, which could be used to generate glycoconjugates vaccine [27,165].

The same analytical comparison made for the O:4,5-antigen extracted using the hot phenol method was done on GMMA recovered from *S. Tyhphimurium* SL1344 *tolR*⁻ fermentation broth and on the biomass from *S. Tyhphimurium* SL1344 wild type, both treated with the direct acid hydrolysis method.

The total sugar quantification by phenol sulfuric assay was done, on both hydrolyzed samples, and a comparable concentration was found, indicating that GMMA are enriched in membrane components like LPS and therefore O-Antigens.

Another important advantage of using GMMA, as O-Antigens source, is related to the purification method after the hydrolysis.

The standard O-antigen purification protocol, proposed by Micoli et al, consists in several steps necessary to remove, from the hydrolyzed material, residual nucleic acids and proteins. Usually the final residual impurities concentration must be less than 1%.

The standard O-antigen purification protocol was applied on the hydrolyzed GMMA samples from *S. Tyhphimurium* SL1344 *tolR*⁻ and on *S. Tyhphimurium* SL1344 wild type biomass, to have a purification processes comparison starting from different materials.

In the hydrolyzed material from *S. Tyhphimurium* SL1344 wild type biomass was found, as expected, a huge proteins and nucleic acids concentration which must be removed during the purification protocol, to obtain the polysaccharide with the expected purity.

In the hydrolyzed material from GMMA, since the beginning, a low amount of impurities was found, especially regard nucleic acid, thus the purification process could be significantly simplified avoiding several costly purification steps, necessary in the purification protocol developed for the biomass.

We tested on the hydrolyzed material of GMMA the standard purification protocol as well as other protocols, removing one or more purification steps. Due to the low percentage of impurities present in the hydrolyzed GMMA, several purification steps were not necessary to obtain the O-antigen with the expected purity. Only the chromatographic step (cationic exchange chromatography) was required to remove all residual proteins.

Therefore, starting from GMMA, as O-antigen source, the purification protocol could be significantly reduced, diminishing time and costs relative to the entire process.

I wanted also check the composition and structure of the O-Antigens obtained from the two different starting materials (GMMA from *S. Tyhphimurium* SL1344 *tolR*⁻ in comparison with the biomass from *S. Tyhphimurium* SL1344 wild type) and using different purification protocols.

At the end of the purification process, the O-antigen from GMMA was analyzed by HPLC-SEC and three different populations were found: O:4.5 antigen High Molecular Weight (HMW), O:4.5 antigen Medium Molecular Weight (MMW) and O:4.5 antigen Low Molecular Weight (LMW). The MMW and the LMW O-antigens populations were much more abundant than the HMW while in the *S. Tyhphimurium* SL1344 wild type biomass, enrichment in terms of HMW was found, respect the MMW population.

The MMW O: 4.5 antigen from GMMA of *S. Tyhphimurium* SL1344 *tolR*⁻ was extracted, purified and further characterized to compare its composition respect the O:4,5-antigen from *S. Tyhphimurium* SL1344 wild type.

In the MMW O: 4,5-antigen from GMMA was found galactose, rhamnose, mannose in the ratio 1:1 as the structure of O:4,5-antigen from the wild type strain . The only one difference was found in the glucosylation level. In the MMW O:4,5 antigen from GMMA was found less glucose (relative to rhamnose) than in the O:4,5 antigen from the wild type strain.

In conclusion:

- GMMA particles could be used as O-antigens source (and in general as source of membrane components) due their membrane composition enriched in LPS;
- O-antigens can be extracted from GMMA using the direct acidic hydrolysis method, avoiding all issues related to the toxicity of the phenol, commonly used for the LPS extraction;
- O-antigen from GMMA is composed predominantly by the Medium Molecular Weight (MMW) population;
- O-antigen from GMMA has the same structure observed in the wild type strain;
- The O-antigen purification process starting from GMMA is much more easy and cost effective than from the biomass.

Using the electron microscopy approach on *S. Typhimurium* SL1344 *tolR*⁻ strain we have capture the nascent GMMA during the bacteria growth.

We have found that, GMMA particles release occurred along cell body and also at the division septa. During the bacterial duplication event, the Tol-Pal complex migrates to the division septa modulating GMMA production and release from this specific point [165,166].

During the *S. Typhimurium* SL1344 *tolR*⁻ GMMA recovery process optimization, reported in the results section, the best condition for high GMMA recovery yield was obtained using the 100KDa cut off TFF membrane, due to the relationship between GMMA size and membrane cut off.

The “GMMA 0.1 μm retentate” sample showed a huge amount of flagella which interact with GMMA, forming a sort of “grid structure”. This TFF approach, useless for GMMA recovery, could be used to purify flagella from the fermentation broth, a similar approach has been already proposed by Oliveira et al. Some molecules could be extracted directly from bacterial flagella, an example of this application is the flagellin protein, which is used in diverse biotechnological applications, such as vaccine adjuvant and cellular protector during chemo-

and radiotherapy (Oliveira H. et al; Brazilian Journal of Chemical Engineering; Vol. 28, No. 04, pp. 575 -584, October - December, 2011)

Another important aspect found using the electron microscopy approach was related to the GMMA encapsulation forming the structure “GMMA inside GMMA”.

In all samples were found small GMMA contained in the inner portion of a big GMMA. The mechanism which leads this phenomenon is unknown but we have some hypothesis: the outer membrane instability promotes the GMMA release so the outer membrane rearrangement could provoke the encapsulation of small GMMA into other GMMA; more GMMA produced from the same outer membrane point interacts each other, fusing their membrane and creating these structures. However, the reason of this phenomenon is already unknown and further experiments are required.

GMMA could be used as vaccines itself, exploiting their properties as immune stimulator, due to the LPS and OM proteins exposition on their external surface. This GMMA peculiarity is very important for vaccines development.

We have no information about the antigen molecules orientation in GMMA produced by *S. Typhimurium* SL1344 *tolR*, especially for small particles encapsulated in the internal space of a bigger one GMMA.

The possible risk related with the structure “GMMA inside GMMA” is the membrane rearrangement which could influence the antigen molecules orientation, switching the external portion of the outer membrane to the opposite side, relocating the outer membrane proteins and LPS in the GMMA inner space, influencing the GMMA-host interaction, with a possible negative impact on vaccines development. To define the outer membrane orientation and to visualize the LPS exposition on our GMMA surface we are currently work on Immune Golden Labeling assay optimized for GMMA preparation.

In literature is already reported that, the site of nascent GMMA release may also manipulate the GMMA protein constituents. Proteins in septal and cell body derived GMMA were identified by LC-MS/MS and quantified. Significant differences in the abundance of specific proteins in each GMMA population were identified at a 90% confidence level. Representative results are reported from two independent experiments demonstrating that specific proteins are differentially represented in GMMA released at septa or from cell body locations. For example, TolB, known to migrate to division septa during constriction was highly enriched in

septal GMMA. Cell body-derived GMMA, however, were enriched in the flagellar cap and hook/filament junction proteins FliD and FlgK [166].

The specific protein enrichment in different kinds of GMMA is another important point, if GMMA particles want to be used as vaccines itself, could be possible define where the target immunogenic protein is expressed and could be possible select, by size, the specific GMMA population. Using this approach could be possible the development of a more specific GMMA based vaccines.

2. Implication for Vaccines Development

The same approach used and developed for *S. Typhimurium* SL1344 *tolR*⁻ could be applied to other gram negative strains to purify membrane components as polysaccharides or proteins. This new method for purification of O-antigens used to develop a glyconjugate vaccine is the connection between two technology platforms: the direct acid hydrolysis approach developed to purify O-Antigens directly from the biomass, and the GMMA particles used as vaccines.

The direct consequence of this new paradigm is the significant decrease of Cost of Goods connected with the entire manufacturing process.

We would develop and facilitate the introduction of new cost effective vaccines to diminish the susceptibility of disadvantaged populations to the predominant enteric pathogens responsible for pediatric mortality and severe morbidity [170,171].

We would promote vaccination against diseases which causes thousands of children death especially in developing countries, like diarrhea which is one of the most common causes of morbidity and mortality in children worldwide [172,173].

GMMA particles could belong in this context.

GMMA could be used not only as membrane component source but also to develop broad spectrum vaccines due to the complexity of the outer membrane molecule.

NVGH is developing a new broad spectrum GMMA based vaccines against *Shigella* spp, exploiting their antigenic properties, using the entire particle to induce an immune response. Clinical trials are still on going to ensure vaccines efficacy and safety.

GMMA particles could be used also in combination with the reverse vaccinology approach [174].

Recent advances in genomics and reverse vaccinology have identified promising protein targets for vaccines. In many cases, suitable candidate antigens for Gram-negative bacterial vaccines are outer membrane proteins and these pose particular challenges in their expression and purification and in serotype variability. GMMA particles could be an ideal delivery system, especially for bacterial vaccines for developing countries, and also could encompass multiple antigens.

We believe that, GMMA technology is an innovative, efficient and cost effective platform for vaccine manufacturing against Gram-negative bacteria moreover this platform is very flexible and it could be exploited applying different approaches, as immunogenic molecules source or as vaccines itself.

Using genetic manipulation, it is possible increase GMMA yield, remove immunodominant structures, overexpress specific antigens and reduce the endotoxic activity thus GMMA could be potentially used as a safe and effective low cost vaccine

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