School of Veterinary Medicine

PhD course in Veterinary and Animal Sciences

(Class XXIX)

Department of Health, Animal Science and Food Safety

PhD Thesis in Veterinary and Animal Science

Molecular farming applied to Veterinary Science: *Nicotiana tabacum* plants expressing antigenic proteins from *Escherichia coli* as a model of edible vaccine in weaned piglets

(SSD AGR/18)

Angela LOMBARDI

University ID: R10528

Tutor: Full Professor Antonella BALDI

PhD course coordinator: Full Professor Fulvio GANDOLFI

Academic Year 2015-2016

“You have to learn the rules of the game. And then you have to play better than anyone else.”

“Devi imparare le regole del gioco. E poi devi giocare meglio di chiunque altro. “

Albert Einstein

ABSTRACT

Medical Molecular farming is a term used to refer to the production of valuable biopharmaceuticals in plants. An important branch of medical molecular farming is represented by edible vaccines: engineered plants expressing major immunogenic proteins of pathogens that can be administered as subunit vaccines by oral route. Edible vaccines are particularly attractive due to low cost, heat stability, avoidance of injections and ability to induce specific antibodies in the mucosa, where enteric pathogens gain access to the body.The chances of acquiring mucosal immunity against infectious agents that enter the body across mucosal surface are also increases with the oral delivery of vaccines.

This study is focused on seed-based vaccines against swine verocytotoxic *Escherichia coli* (VTEC). The third generation (R_3) of two lines of *Nicotiana tabacum* expressing, respectively, the F18 adhesive fimbriae (n: 80) and the B subunit of the VT2e toxin (n: 80) of *E. coli*, were harvest in contention conditions (level 1). Four hundred and seventy grams of F18 positive seeds (F18s) and four hundred and fifty grams of VT2e-B positive seeds (VT2eBs) were collected. Even if no differences were observed in the germination index (F18s: 73.3%; VT2e-Bs: 76.6%; WT: 78.5%), F18s and VT2eBs germinated four days later than the wild type. This aspect was probably related to late metabolic employment of inclusion bodies that we observed in the vaccinal seeds by morphological analyses. Polymerase chain reaction, carried out on DNA from vaccinal plants, confirmed the stable integration of the bacterial-origin genes into plant genome. The amount of F18 and VT2e-B was estimated respectively about 6.6-7.4 μg and 34-37 μg per 10 g seeds respectively by competitive indirect ELISA. The protective effect was evaluated, through an *in-vivo* trial, carried out at the Experimental Animal Research and Application Centre of the University of Milan, on 36 weaned piglets (controls, CG, n:18; treatment, TG, n:18) fed 20 g vaccinal seeds (10 g of each line) mixed with 40 g of milk powder on days 0, 1, 2, 5 and 14. Controls received wild type tobacco seeds in the same way. 12 piglets per group were challenged at day 20 with 10^{10} CFU of O138 *E. coli.* Throughout the experimental period (0-29 days), zootechnical performances and health status were monitored and biological

samples were collected individually. After the immunization period (day 20th), the titer of total intestinal IgAs resulted higher in the treated piglets, which showed also a significant increase of the fecal anti-F18 and anti-VT2e-B Immunoglobulin-A, if compared with controls. The adopted oral delivery strategy appeared effective in reducing clinical signs after VTEC infection and was able to stimulate local immunity in gastrointestinal tract.

Finally, the knowledge in mucosal immunity was deepened in collaboration with the Department of Infectious Diseases, University of Denver, Colorado.

In particular a method for characterizing the effects of different mucosal bacterial pathogens such as *Moraxella catarrhalis,* (Mcat), *Heamophilus influenzae* type b (Hib)*, Haemophilus influenzae* non typeable (NTHi*), Staphilococcus aureus Cowan I* (SAC) on the isotype of antibody production from human B cells, was set up.

RIASSUNTO

"Medical Molecular farming" o "Agricoltura molecolare" è un termine usato per indicare la produzione su larga scala di molecole ad interesse medico o farmaceutico in pianta. In tale ambito un'importate attenzione è rivolta ai vaccini edibili: piante ingegnerizzate per l'espressione di proteine immunogene in grado di indurre, dopo somministrazione orale, una risposta immunitaria locale protettiva nei confronti del patogeno contro il quale sono state progettate.

I vaccini edibili offrono numerosi vantaggi correlati alla facilità di somministrazione, ai bassi costi, alla facilità di stoccaggio nonché alla possibilità di indurre una risposta immunitaria locale laddove il patogeno ha accesso nell'organismo.

Questo studio si concentra sui semi vaccinali contro i ceppi *Escherichia coli* verocitotossico (VTEC) nei suinetti. La terza generazione (R_3) di semi di due linee di *Nicotiana tabacum* che esprimono, rispettivamente la fimbria adesiva F18 (n: 80) e la subunità B della tossina VT2e (n: 80) derivanti da un ceppo di *E. coli* verocitotossico, sono state coltivate in serra con un livello di contenimento 1. Nello specifico, sono stati raccolti quattrocentosettanta grammi di semi F18 positivi (F18s) e quattrocentocinquanta grammi di semi VT2e-B positivi (VT2eBs). Anche se, non sono state osservate differenze nell'indice di germinazione (F18s: 73,3%; VT2e-B: 76,6%; WT: 78,5%), i semi F18s e VT2eBs hanno germinato quattro giorni più tardi rispetto ai semi di *Nicotiana tabacum* "wild type". Questo aspetto è probabilmente correlato all'uso tardivo dei corpi di inclusione (proteine e lipidi) da parte delle varietà ingegnerizzate, come osservato nei tessuti vegetali attraverso analisi morfologiche con microscopia ottica ed elettronica. Le analisi di PCR, effettuate su DNA di piante vaccinali, hanno confermato l'integrazione stabile dei geni di origine batterica nel genoma vegetale. La quantità delle proteine F18 e VT2e-B è stata stimata essere rispettivamente circa 6,6-7,4 µg e 34-37 µg per 10 g di semi tramite saggi ELISA competitivo indiretto. L'effetto protettivo della somministrazione orale dei semi vaccinali nei confronti con ceppi VTEC è stato valutato, attraverso una sperimentazione *in-vivo*, effettuata presso il Centro Zootecnico Didattico Sperimentale dell'Università degli Studi di Milano. Nello specifico 36 suinetti

svezzati a 21 giorni sono stati suddivisi casualmente in due gruppi sperimentali: controllo (CG, n:18) e trattamento (TG, n:18). Considerando l'inizio della sperimentazione il giorno 0, i soggetti del gruppo TG hanno ricevuto nei giorni 0, 1, 2, 5 e 14 un trattamento corrispondente a 20 g di semi vaccinali macinati miscelati con 40 grammi di latte ricostituito in polvere. Il gruppo controllo ha invece ricevuto 20 g di semi di tabacco wild type. Al giorno 20, 12 suinetti rispettivamente del gruppo controllo e del gruppo trattamento sono stati infettati sperimentalemente con 10^{10} CFU del sierotipo O138 di *E. coli.* Durante tutto il periodo sperimentale (0-29 giorni), performances zootecniche e stato di salute sono stati monitorati e sono stati raccolti individualmente campioni biologici. Dopo il periodo di immunizzazione (giorno 20), il titolo di IgA intestinali totali è risultato essere superiore nei suinetti trattati, che inoltre mostravano un significativo aumento del titolo fecale di immunoglobuline A anti-F18 e anti-VT2e-B rispetto ai controlli. La strategia adottata, tramite somministrazione orale è risultata efficace nel ridurre i segni clinici dopo l'infezione da VTEC ed è stata in grado di stimolare l'immunità locale nel tratto gastroenterico. Inoltre, la conoscenza delle tecniche immunologiche e un approfondimento sull'immunità mucosale locale sono state sviluppate in collaborazione con l'Università di Denver, Colorado. In particolare è stato messo a punto un metodo per caratterizzare gli effetti di diversi batteri patogeni mucosali, come *Moraxella catarrhalis,* (Mcat), *Heamophilus influenzae* di tipo b (Hib), *Haemophilus influenzae* non tipizzabile (NTHi), *Staphilococcus aureus Cowan* I (SAC*)* sulla produzione di anticorpi di cellule B umane

DETAILED CONTENTS

1-INTRODUCTION 11

1.1-Molecular Farming 11

1.2-Selection of the host species 14

1.3-Plants transformation: an overview 16

1.3.1-Stable expression in transgenic plants 18

1.3.2-Transient expression in transgenic plants 22

1.4-New advances in crop genetic engineered tools 26

1.5-Plastid transformation 29

1.6-Optimization of the expression in host plants 31

1.6.1-Optimization by Codon Usage 32

1.6.2-Synthetic biology 33

1.6.3-Optimization of Promoters 35

1.6.4-RNA-processing, intron mediated enhancement and stability 36

1.7-Targeting protein 37

1.8-Seed-based platforms 37

1.9-Suitable host and model organism in Molecular Farming 39

1.9.1-*Arabidopsis thaliana* 39

1.9.2-Cerals and legumes 40

1.9.3-Vegetables and fruits 41

1.9.4-*Nicotiana tabacum* 42

1.10-Edible vaccines 44

1.11-Challenge of plant-based vaccines 46

1.11.1-Selection of antigen and plant expression host 47

1.11.2-Constistency of dosage 47

1.11.3-Manufacturing of vaccines according to GMP procedures 48

1.12-Edible vaccines in Veterinary Medicine 51

1.12.1-Plants based vaccines against parasitic diseases 54

1.12.2-Plant based-vaccines (PBV) against bacterial or viral diseases 56

1.12.3-PBV against zoonotic diseases 57

1.12.4-PBV specie-specific pathogens 60

1.13-*Escherichia coli* infection in pig livestock 63

1.13.1-O138 verocytotoxic *E. coli* in weaned piglets 64

2-AIM OF THE STUDY **70**

3-MATERIALS AND METHOD **71**

3.1-Vaccinal biomass production 72

3.1.1- Seeding 72

3.1.2- Germination and morphology evaluation 73

3.1.3-Cultivation in the greenhouse 73

3.2-Seeds collection 74

3.3-Chemical analisyses of principal components of vaccinal tobacco seeds 75

3.4-Molecular analyses of tobacco plants 75

3.4.1-DNA extraction from seeds and leaves 75

3.4.2-Detection of F18, VT2e-B, NOS genes 76

3.5-Evaluation of the expression of antigenic proteins in seeds 77

3.5.1-Positive control: purified antigens 77

3.5.2-Specific antibodies against antigens 78

3.5.3-Set-up ELISA assay 79

3.5.4-Quantification of F18 in vaccinal seeds 79

3.5.5-Quantification of VT2e-B in vaccinal seeds 80

3.6-Microscopical evaluation of plants 81

3.7-*In-vivo* trial: evaluation of oral administration of tobacco seeds as edible vaccine in weaned piglets 81

3.7.1-Animals, housing conditions 81

3.7.2-Experimental design 84

3.7.3-Treatments 84

3.7.4-Challenge 85

3.7.5-Sample collection and zootechnical evaluation 86

3.8-Evaluation of intestinal Immunoglobulin-A titer in the immunization phase 87

3.8.1-Evaluation of total Immunoglobulin-A during immunization phase 87

3.8.2-Evaluation of anti-F18 IgAs and anti-VT2e-B IgAs in fecal samples 88

3.9-Statistics 89

4-RESULTS AND DISCUSSION 90

4.1-Production of tobacco seed-based oral vaccine against verocytotoxic *Escherichia coli* (VTEC) 92

4.1.1-Cultivation and seeds collection 92

4.2-Chemical analyses of principal components of vaccinal tobacco seeds 96

4.3-Molecular analyses of tobacco plants 98

4.4-Morphological evaluation of plants 99

4.5-Expression and quantification of antigenic proteins in tobacco seeds 101

4.6-*In-vivo* trial: evaluation of the oral administration of tobacco seeds as edible vaccine in weaned piglets 104

4.6.1-Strategical optimization of vaccinal tobacco seeds administration 104

4.6.2-Zootechnical performances 106

4.6.3-Evaluation of clinical signs 109

4.7-Evaluation of intestinal Immunoglobulin-A titer during the immunization phase 111

4.7.1-Evaluation of anti-F18 IgAs and anti-VT2e-B IgAs in fecal samples 113

5-CONCLUSIONS 116

6-SUPPLEMENTARY STUDY 120

6.1-Isotypes distribution of antibodies in response to T-cells independent stimulation 120

6.1.1-Mucosal microbiota 120

6.1.2-Lymphoid organs of the respiratory tract 122

6.1.2.a-Structure of NALT (Nasal associated lymphoid tissue) 123

6.1.3-Function of NALT 124

6.1.4-The B cell development 125

6.1.4 a-B-cell developmentand immunoglobulin class switching 125

6.1.5-Outline of molecular mechanism for class switching recombination (CSR) 127

6.1.5.a-Role of AID in class switching recombination 128

6.1.6-The human mucosal B-cell system 129

6.1.6.a-Microbial impact on mucosal B-cell 132

6.1.7-Bacterial interaction with mucosal epithelial cells 132

6.1.7.a- Bacterial interaction with epithelial cells in the respiratory tract 132

6.1.8-Briefly overview of microorganism of the respiratory tract 135

6.2-Aim of the study 138

6.3-Materials and method 139

6.3.1-Bacteria and culture conditions 139

6.3.2-Formaldehyde killing assay 140

6.3.3-Reagents 140

6.3.4-Culture cells and samples 141

6.3.5-B-cells stimulation (Stimulation assay) 142

6.3.6-Enzyme–linked immunosorbent assay (ELISA) 143

6.3.7-Statistics 145

6.3.8-Flow cytometer analysis 145

6.4-Results and discussion 146

6.4.1-Bacteria culture 146

6.4.2-Stimulation assay 147

6.4.3-Flow cytometer analysis 154

6.5 Conclusions 158

7-REFERENCES **162**

Acknowledgements **193**

1-INTRODUCTION

1.1-Molecular Farming

The term Molecular farming generally refers to the production of pharmaceutically important and commercially valuable proteins in plants (Fischer and Emans, 2000). Historically, plants were used as an exceptionally valuable source for treatments and therapies. Thousands of plant species have been utilized for medicines and most of the world's population employs them to treat acute and chronic health complications. After a change from wild collection to cultivation, improvements in plant biotechnologies, which occurred over a long period, aimed to directly improve and modify specialized elements of plants such as carbohydrates, proteins, oils, fats and vitamins. Nevertheless, the use of engineered plants as "bioreactors" is a quite new bioscience and is achieving momentum (Sharm and Sharma, 2009).

Utilizing transgenic plants for the fabrication of high-value recombinant proteins for industrial and clinical purposes has become a promising substitute to usual bio-production systems, like bacteria, yeast, and cultured insects and animal cells. This innovative system has numerous advantages compared to conventional systems such as their safety, scale, cost-effectiveness, ease of distribution and storage (Table 1).

Comparisons	Transgenic Plant	Plant Cell Culture	Bacteria	Yeast	Mammalian Cell Culture	Transgenic Animals
Overall cost	Very low	Medium	Low	Medium	High	High
Scale-up capacity	Very high	Medium	High	High	Very low	Low
Production scale	Worldwide	Limited	Limited	Limited	Limited	Limited
Protein yield	High	High	Medium	High	Medium-High	High
Protein folding accuracy	High	High	Low	Medium	High	High
Glycosylation	Minor differences	Minor differences	None	Incorrect	Correct	Correct
Product quality	High	High	Low	Medium	High	High
Contamination risks	Low	Low	Endotoxins	Low	Virus, Prions, oncogenic DNA	Virus, Prions, oncogenic DNA
Safety	High	Non-specific	Low	Unknown	Medium	High

Table 1: Comparison of different expression platforms for the production of pharmaceuticals (modified from Spök and Karner, 2008).

Plants can be sources of pharmaceutical proteins, such as blood-component substitutes (Magnuson et al., 1998), mammalian antibodies (Fischer et al., 1999a), and vaccine subunits (Walmsley and Arntzen, 2000). By using engineered plants, scientists succeeded in incorporating genes into host plants that were subsequently induced to generate pharmaceuticals (Torrent et al., 2009b). Barta et al. (1986) have produced the first plant-based medical protein during their studies, generating a human growth hormone in tobacco (*Nicotiana tabacum* spp).

Although the idea of creating human proteins and antibodies in plants was at first seen with skepticism, crops showed to offer a unique combination of advantages as well as remarkable features compared with those of the conventional production systems. During the last decades, plants generally have been extensively investigated as unconventional systems for the production of pharmaceuticals proteins.

Easy and cheap to grow. Plants possess excellent biosynthetic capabilities including the ability to use the sun energy (photosynthesis) and/or very simple media to support significant biomass and protein accumulation. They have a large potential for low-cost production of high quality and bioactive recombinant proteins (Sharm and Sharma, 2009; Obembe et al., 2011; Paul and Ma, 2010). In the near future, the world's population and the request for cheap end effective medical products will grow once again; plants will be the main help for humans and animals. Compared to conventional systems, plants are cheap and harmless. As showed in Daniell et al. (2001b), the cost of monoclonal antibodies produced with the hybridomas method is 1000$/gram and 100$/gram when using transgenic animals but when using transgenic plants, the productions cost decreases to 50$/gram.

Safety. Safety, for humans and animals, is important to avoid outbursts of infections and diseases; plants decrease health risks from pathogens cross-contaminations. Plants are not infected by possible human pathogens, such as bacteria, prions or viruses, which reduces production costs, and minimizes health dangers.

Features and limitations of traditional production systems. To supply the extensive demand of medical proteins, the pharmaceutical industry industrialized some production systems. Plants classically produce recombinant proteins with the correct folding activity and glycosylation (Schillberg et al., 2005; Yian et al., 2015). For instance, targets for specific human recombinant antibodies are recognized in the clinic as in the therapeutics and denote a major new class of drugs. Therapeutic effectiveness depends on the development of complexes with target molecules and consequent activation of downstream biologic mechanisms, which result in the removal of the target. The activation of effector mechanisms depends on the characteristics of the structure of the antibodies molecule that derives from post-translational modifications, in particular, from glycosylation. The production of curative antibodies with a stable glycoform profile is a significant challenge for the biopharmaceutical industry. Indeed, bacteria do not perform glycosylation of heterologous proteins, yeasts as *Pichia pastoris* and *Saccharomices cerevisiae* lead to hyper-glycosylation, no sialyltransferase activity. Instead, insect cells generate extensive glycosylation, no sialyltransferase activity and insect specific glycans, could be unsafe (*e.g.* bee venom-related to anaphylactic shock). Mammalian cell cultures are pricy and raise concerns about safety. Glycosylation outlines depend on cell lines and culturing circumstances. Plants and their glycol engineering confer naturally closer human glycan's (Table 2). However, broad engineering could be limited due to serious side effects of altering glycosylation patterns of endogenous proteins. In addition, plants do not generate endotoxins (unlike bacteria) and they do not support replication of human viruses (on the contrary of mammalians cells).

N-glycosylation in mammal cells *versus* plants	
Mammals	**Plants**
▪ α1,6 core fucosylation ▪ proximal 1,4 galactosidation ▪ terminal sialylation ▪ complex glycans dominate	• α1,3 core fucosylation & β1,2 core xylose • proximal galactosidation not common, • only 1,3 type proximal α1,3 fucosylation • no terminal sialylation

Table 2: Foremost differences in term of N-glycosylation pathway between mammal and plant cells. Plants do not generate endotoxins (unlike bacteria) and they do not support replication of human viruses (on the contrary of mammalians cells).

The precise control of glycosylation permitted the production of plant-derived glycoproteins with humanlike or human-compatible glycans, as well as biobetters (the term biobetter indicates a recombinant protein drug that is in the same class as an existing biopharmaceutical but is not indistinguishable), in which the glycan profiles have been poked to improve efficacy or longevity, or to make downstream processing easier.

The technology is now accessible for harvesting and processing plants and plant products on a large scale. The quantity of recombinant product that can be produced approaches industrial-scale levels. The purification requisite can be escaped when the plant tissue, which contains the recombinant protein, is used orally. Plants can be guided on target proteins in intracellular compartments in which they are more stable, or even to express them straightforwardly in certain compartments (chloroplasts, seeds).

1.2-Selection of the host species

For the effective production of recombinant product, selection of the host species is essential. Even in the beginning, the *Arabidopsis thaliana* and the *Nicotiana tabacum* models, which are easy to transform and manipulate, were the main system for the production of most of the plant-derived recombinant proteins. For this purpose, today a large number of plant species are being used such as tomato, banana, rice, maize, wheat, carrot, soybean, pea, potato, lettuce and *alfa-alfa* (Kamenarova et al., 2005). A large variety of products can be created in plants, but each one has its own specific necessities for its production. The choice of the host species is associated with the type of protein in question *i.e.* the recombinant protein form, which will be produced at the end (Table 3). Together with the technical improvements, a huge number of plant species attracted the attention of the scientific community. Generally, domesticated species are chosen over wild species for molecular farming since they are adapted to a broad range of environmental conditions because of their commercialization. Yet, wild species could be more strategic as they will not be mistaken for food crops and would report the issue of food crop mix-up with transgenic products.

Plants	Advantages	Disadvantages
Tobacco	Efficient transformation system. Abundant material for protein characterization.	Toxic alkaloids. Potential for outcrossing in field.
Arabidopsis thaliana	Efficient transformation system. The genome sequence was completed in 2000. Extensive EST resources are available.	Not suitable for cultivation on an agricultural scale.
Potato	Efficient transformation system. Tuber is edible raw. Tuber specific promoters available. Clonally propagated.	Relatively low tuber protein content. Unpalatable raw form cooking may cause denaturation and poor immunogenicity of vaccines.
Tomato	Relatively efficient transformation system. Fruit is edible raw. Fruit specific promoter is available. Industrial fruit processing well establishes. Industrial greenhouse culture well establish.	Relatively low fruit protein content. Acidity fruit may be incompatible with some antigen or for delivery to infants. No *in vitro* system to test fruit expression.
Alfa-alfa	Relatively efficient transformation system. High protein content in leaves. Leaves edible uncooked. Ideal system for animal vaccines.	Potential for outcrossing in field. Deep root system problematic for cleaning in field.
Legumes and cereals	Production technology well establish. High protein content in seeds. Stable protein in stored seeds. Well suited for animal's vaccines. Industrial seed processing well establishes.	Inefficient transformation system. Heating or cooking for human use may cause denaturation poor immunogenicity for vaccines. Potential for outcrossing in field for some species.
Banana	Cultivated widely in developing countries (vaccines are needed). Eaten raw by infants and adults. Clonally propagated. Once stablish, abundant end inexpensive fruit is available on 10-12 months cycle.	Inefficient transformation system. Little data available on gene expression especially for fruit specific promoters. High cultivation space requirement, expensive in greenhouse.

Table 3: Features for different plants host system (by Warzecha and Mason, 2003)

The production of plant-derived pharmaceuticals has attracted huge interest for their industrial development. Some achievements of MMF are listed in table 4.

Product	Host	Application	Clinical Trial	Status	Sponsor
Taliglucerase alfa; Recombinant glucocerebrosidase (prGCD)	Carrot cell culture	Gaucher disease	NCT00376168	Phase 3 completed (2012); FDA approved (2012)	Protalix, Karmiel, Israel
ZMApp	Tobacco	Ebola Virus	NCT02363322	Phase 1 and 2 (2015)	National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD, USA
Vaccine Pfs25 VLP	Tobacco	Malaria	NCT02013687	Phase 1 (2015)	Center for Molecular Biotechnology, Plymouth, MI, USA
Vaccine Recombinant protective antigen	Tobacco	Anthrax	NCT02239172	Phase 1 (2014)	Center for Molecular Biotechnology, Plymouth, MI, USA
HAI-05	Tobacco	H5N1 Vaccine	NCT01250795	Phase 1 (2011)	Center for Molecular Biotechnology, Plymouth, MI, USA
Recombinant human intrinsic factor	*Arabidopsis thaliana*	Vitamin B12 deficiency	NCT00279552	Phase 2 completed (2006)	University in Aarhus, Aarhus, Denmark

Table 4: Plant-derived pharmaceuticals in clinical trials, *modified from U.S. National Institutes of Health Clinical Trial (2016).*

In particular, some success stories are mentioned below.

Mapp Biopharmaceutical Inc., a company positioned in San Diego, CA, USA produced a drug in tobacco leaves named ZMapp, which has been used to fight the 2014 Ebola virus outbreak in Africa (Arntzen et al., 2015). By October 2014, seven infected patients got an early treatment with ZMapp and recovered. However, another patient, receiving a late treatment with ZMapp in November 2014, encountered the disease and died. Further Ebola patients were unable to receive the treatment due to an insufficient stock of ZMapp. This is unfortunate as it is the only drug up to date that has been effectively used to treat patients infected with the Ebola virus, even though it has not been officially accepted by the U.S. Food and Drug Administration (FDA). ZMapp underwent a clinical Phase I and two trials in 2015, sponsored by the National Institute of Allergy and Infectious Diseases (NIAID).

Middle East respiratory syndrome coronavirus (MERS-CoV) is a developing illness. Due to the high mortality rate of MERS (above 35%), it caused a public panic in South Korea during May 2015. By 27 November 2015, MERS-CoV infected 1618 patients and caused 579 deaths globally. Over 26 countries reported MERS-CoV cases. Today, still no effective drug is available to treat the MERS-CoV virus. Plant Biotechnology Inc. (Hayward, CA, USA) produced an immune-adhesin (DPP4-Fc) in transgenic tobacco. Purified DPP4-Fc presents a strong binding to MERS-CoV and inhibits the virus from infecting lung cells. In June 2015, Plant Biotechnology Inc. received funding’s from NIAID to support further development and testing of this.

1.3-Plants Transformation: an overview

Plants transformation indicates the introduction and integration of endogenous DNA into the plants genome and the consequential expression of exogenous genes. Today, the two main expression targets are considered seeds and leaves. The two major leafy crops used for the production of recombinant proteins are tobacco and *alfa-alfa*, which both have a high leaf biomass yield because they can be cropped more times a year. The principal drawback of leaf transformation is that the harvested leaves generally have a restricted shelf life. The recombinant proteins endure in a humid situation and are therefore somewhat unstable, which

can decrease product yields, the benefit of seed transformation is that recombinant proteins can be directed to accumulate specifically in the dried seeds. Therefore, although seed biomass yields are lower than the leaf biomass yields of tobacco and *alfa-alfa* this is balanced by the increased stability of the proteins. Seeds are natural storage organs, with the best biochemical environment for the accumulation of large amounts of proteins. The accumulation of proteins in the seed rather than vegetative organs also precludes any toxic effects on the host plant. Finally, the extraction of proteins from seeds is enabled because the target protein is condensed in a small volume; most cereal seeds lack the phenolic compounds that are often found in leaves and which interfere with processing, and the seed proteome is quite simple, which reduces the possibility of contaminating proteins.

Transfer DNA into plants cells can result in a transient or stable expression of the introduced DNA.

<u>Stable transformation</u>, is often a long process implicating tissue culture techniques that facilitate the growth of whole plants from treated cells or tissue explants. Because of its stable transformation, the introduction of DNA is integrated into the DNA of the host cell and is thereby suitable to be pass on to the next generation (Newell, 2000).

<u>Transient expression</u>, as his name advocates, usually lasts for only a few days, and represents a useful tool in such areas as development of transformation methodology or metabolic studies since it permits the effects of experimental manipulation to be seen quickly.

Both stable transgenic plants (Shinmyo and Kato, 2010*)* and plant-based transient expressions methods (Komarova et al., 2010*)* have been used to produce recombinant peptides. Characteristically, transient expression produces higher yields and allows more rapid accumulation, whereas transgenic plants can provide an almost limitless source of uniform material expressing the target molecule (Obembe et al., 2011). Different features need to be considered the DNA’s size, the time and the economic supplies needed, the transformation efficiency, the rang of transformable plants species, the type of explants of the host organism to realize the transformation.

Plant-cell-suspension culture

Like mammalian cell culture, it is possible to use plants cell suspension, to produce several different biopharmaceuticals. Plants cell suspension is a convenient substitute in molecular farming, considering a single purification practice and easygoing downstream processing procedure. However, a high level of sterility is needed in order to avoid contamination. Moreover, compared to a plant-cell culture system, a plant cell suspension does not require a regeneration procedure; this is essential to guarantee a fast production of molecules. Despite having numerous advantages, plant-cell suspension cultures have been used for few botanical species, *e.g.* Tobacco, carrot, *Arabidopsis* and rice. Besides, this system presents an increasing proteolytic activity, which leads to a low concentration of the recombinant proteins, during the late stationary phase. Based on these concerns, plants cell suspension remains a challenging method for protein expression (Obembe et al., 2011).

1.3.1-Stable expression in transgenic plants

Different approaches can be used for a stable expression of recombinant proteins into plants tissues. Stable transformation of plants is reached mainly through agro-infection or through a biolistic method. However, *Agrobacterium tumefaciens* T-DNA normally integrates into random sites of the plants genome.

Agrobacterium tumefaciens

Agrobacterium tumefaciens is a soil pathogen that colonizes injured plant cells and induces the development of a tumor (or crown gall) that creates special amino acids byproducts called opines, which the bacteria are able to use as a carbon and nitrogen source. The capacity of virulent *Agrobacterium* strains to stimulate tumor growth and opine synthesis, and the capacity to use opines, is given by a resident tumor-inducing plasmid (Ti-plasmid). During the colonization process, a segment of DNA from this plasmid called T-DNA is shifted to the plants nuclear genome. The T-DNA encodes enzymes that synthesize auxins and cytokinins, which brings to an unregulated cell proliferation, and enzymes that synthesize opines from standard amino acids (Gelvin et al., 2003). The Ti plasmid is a naturally occurring vector for plant transformation, but wild type Ti-plasmids are not appropriate vectors for genetic

engineering in plants since they are too large to manipulate, and the oncogenes contained in the T-DNA because uncontrolled proliferation of transformed plant cells and prevent efficient regeneration. The T-DNA must therefore be moved to a smaller, more suitable vector, and neutralized by deleting the oncogenes, and including the appropriate molecular construct. A marker gene must also be incorporated to allow transformed cells to propagate. T-DNA transfer is controlled by approximately 30 genes situated in a separate virulence (vir) region of the Ti plasmid, and these must be supplied in trans using a binary vector system if the T-DNA is placed on a smaller plasmid. Contemporary binary vectors comprise numerous unique cloning sites within the T-DNA, a *lac*-Z marker gene for blue-white selection of recombinants, and a choice of selectable markers to recognize the transformed plant cells (Hellens et al., 2000). The T-DNA is surrounded by a 25-base-pairs (bp) imperfect direct repeats known as T-border sequences, which are not transferred to the plant genome intact, but they are needed for the transfer process. T-DNA transfer is interceded by the *virA* and *virG* gene products, which transduce external signals and activate other vir genes resulting in the construction of a pilus for DNA transfer, and the release of the T-DNA by an endonuclease comprising the products of the virD1 and virD2 genes (Figure 1).

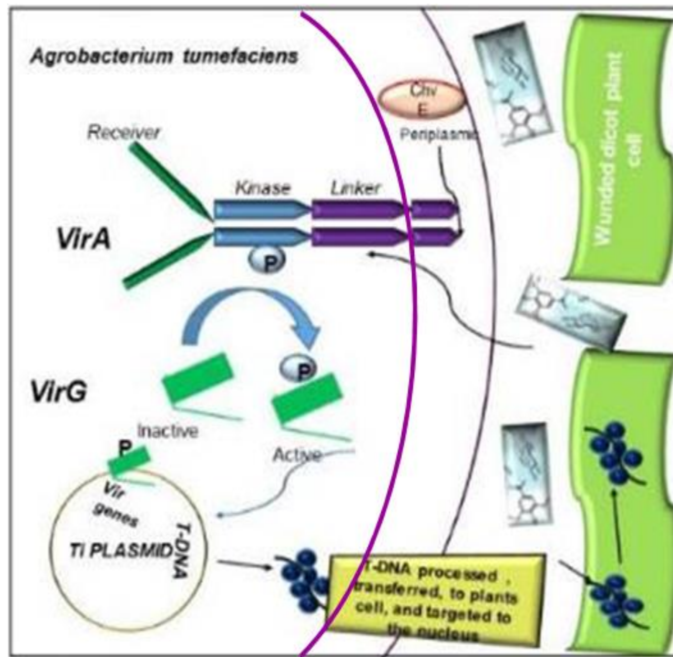


Figure 1: Scheme representing agroinfection process.

A comparative analysis in *Arabidopsis* and rice revealed that T-DNA inserted randomly in the *Arabidopsis* genome (which is globally gene-rich, with little repetitive DNA) but homed in on 10–20% of the rice genome known to be rich in genes, while escaping the more prevalent heterochromatic regions (Barakat et al., 2000). It has also been advocated that T-DNA integration occur favorably in regions displaying micro-homology to the T-DNA borders (Matsumoto et al., 1990), which may also be enriched in the transcribed part of the genome. *Agrobacterium*-based transformation methods have proven to be useful approaches to genetically modified plants of various levels, including model plants such as *Medicago truncalata, Arabidopsis thaliana, Nicotiana tabacum, Nicotiana banthamiana.* Nonetheless, wild-type *Agrobacterium tumefaciens* is a pathogen of dicotyledonous species and the effectiveness of the *Agrobacterium*–based genetic transformation of monocotyledonous plants is still restricted due to the little integration percentage (Abiri et al., 2016). Novel approaches were developed to increase transformation efficiency in monocotyledonous such as maize, rye, barley wheat and rice.

Biolistics

Particle bombardment, or biolistics, is a frequently used method for genetic transformation of plants and organisms. Millions of DNA-coated metal

particles are shot at target cells or tissues by a biolistic device or gene gun. The DNA elutes off the particles that reside within the cells, and a part may be stably incorporated in the host chromosomes (Kikkert et al., 2005). This physical direct technique is used for the genetic transformation of several organisms. Plants transformation using particle bombardment follows the same steps as the *Agrobacterium*-mediated transformation method:

- Isolation of chosen genes from the source organism
- Development of a functional transgenic construct including the selected gene of interest ; promoters to drive expression; modification of the codon, if needed to increase successful protein production and marker genes to facilitate tracking of the introduced genes in the host plants
- Insertion of the transgenic construct into a useful plasmid
- Introduction of the transgenes into plants cells
- Regenaration of plants cells
- Testing of the performance of the traits or gene expression under *in-vitro*, greenhouse or field conditions.

In the particle bombardement method, 1-2 mm tungsten or gold particles (called microprojectiles) coated with genetically engineerd DNA are enhanced with air pressure at high speeds and shot into plant tissues on a Petri-plates, as shown in Figure 2.

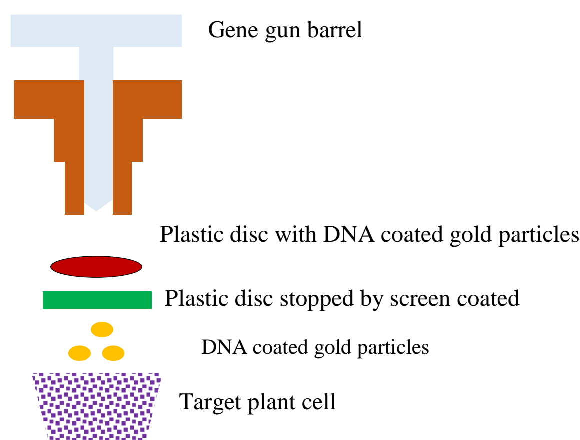


Figure 2: Schematic representation of biotistic tool for plant transformation

This is the second most used method, after *Agrobacterium* mediated transformation, for plants genetic transformation. The device accelerates particles in one of the two ways: by means of pressurized helium gas or by the electrostatic energy released by a dropet of water exposed to high voltage.

Particle bombardment methods are also useful in the transformation of organelles, such as chloroplasts, which enable engineering of organelle-encoded herbicide or resistance in crop plants and to study photosynthetic processes. Restrictions to the particle bombardment method, compared to *Agrobacterium* mediated transformation include frequent incorporation of multiple copies of the transgene at a single insertion site, rearrangement of the inserted genes and insertion of the transgenes at multiple insertion sites. These multiple copies can be associated with the silencing of the transgene in subsequent progeny. The target tissue may often be damaged due to lack of control of the bombardment velocity.

The microcarriers (or microprojectiles), the tungsten or gold particles coated with DNA are inserted by macrocarriers wich are then introduced into the apparatus and pushed downwards at high speeds. The macro-projectiles are stopped by a perforation plate, allowing the microprojectiles to be propelled at a high speed into the plants cells on the other side. As the micro-projectiles enter the plants cells, the transgenes are freed from the particles surface and can be inserted into the chromosomal DNA of the plant cells. Selectable markers give the opportunity to identify those cells that take up the transgene or are transformed. The transformed plant cells are then regenerated and developed into whole plants by using the tissue culture technique.

1.3.2-Transient expression in transgenic plants

Another useful method to obtain biopharmaceuticals from plants is called transient transformation. This method is faster compared to stable transfection, considering that it often takes six months to a year or more to produce transgenic plants. In addition, several generations are required to generate plants that are homozygous for the transgene. Most transformation technologies also result in the gene being inserted randomly into the plants genome. By using the transient transformation, a large quantity of biopharmaceuticals can be created in less than

five days (Andrews et al., 2005; Catrice et al., 2015); especially when compared to the stable transformation method.

Scientists joining and viruses achieved one of the most efficient methods to produce biopharmaceuticals in plants in a short time. The key of this method is to transfer specific components of the viral expression platform by mixing a dissimilar *Agrobacterium* strain harboring portions of the viral machinery, with recombination occurring within the cells once infection has occurred. The well mixing between viral codons and classic eukaryotic introns leads to a significant increase in efficiency of gene delivery and the required amount of the essential infectious *Agrobacterium* reduces. Transient transfection has been developed for small or medium-scale PMF (plant molecular farming) production.

For example, Mason et al. (2010) developed a highly efficient, bean yellow dwarf virus (BeYDV) based single-vector DNA replicon system, which incorporated multiple DNA replicon cassettes. They produced 0.5 mg of antibody per gram leaf (fresh weight) in tobacco leaves within four days following infiltration (Figure 3)

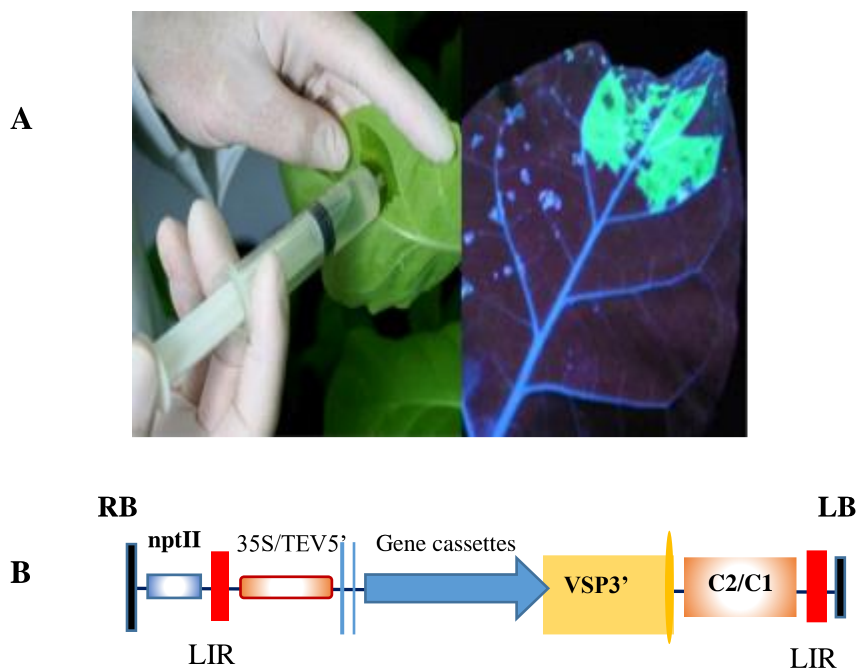


Figure 3 A: Plant-based transient expression system. A: Infiltration of *Agrobacterium* carrying the GFP transgene (Left); and transient expression of GFP (Right). Infiltrated leave can be examined with a UV lamp at four days' post infiltration. B: Diagram of the pBY030.2R vector used by Mason et al. (2010) for the transient expression of GFP.

Mapp Biopharmaceutical Inc. using similar methodology, transiently expressed the humanized antibodies, MB-003 and ZMab, in tobacco leaves. MB-003 and ZMab were later combined and designated as ZMapp. The use of these antibodies as a pharmaceutical drug cured 100% of Ebola infected rhesus macaque's primates (Huang and Arzten, 2010) as previously described. Based on the latter, it is clear that this method is useful, productive and cheap compared to other systems. For example, 1 liter of a bacterial overnight culture could be used to infect about 1000 kg of tobacco leaf tissue, yielding up to 4 kg of recombinant protein at 40% TSP (Marillonnet et al., 2005). This manner, which has just been discovered, to maximize protein yields while reducing the input costs, results in an expected $1/kg of recombinant protein or $50/kg of purified protein (Abiri et al., 2016). Generally, transient expression has two main problems, those being the high technical requirements for the induction and the high risk of accidentally spreading the infection to wild species. In addition, this system remained restricted by laborious scale-up and by controlled conditions, such as those of a greenhouse or laboratory.

<u>Root-absorption.</u>

The pharmaceutical proteins can be efficiently expressed in plants *via* virus vector infection. However, previously mentioned issues prevent development and exploitation for the industrial –scale expression of heterologous proteins.

In a recent paper, the authors described a simple system named "root absorption" to effectively express foreign proteins in plants (Yang et al., 2008). It has been shown that the green fluorescence protein (GFP) was expressed in tobacco plants by root absorbing the *Agrobacterium* suspension containing the TMV-based P35S-30B-GFP vector. Various factors influencing the gene expression were studied including *Agrobacterium* cell density, seedling age, plant materials and inoculation conditions. This system benefits of advantages such as simple and convenient work processes, ease to scale-up and higher level of expression than leaf infiltration. Remarkably, GFP was expressed at 24h post-absorption. We assume that the root absorption system will ease the large-scale production of the recombinant pharmaceutical proteins in plants by means of transient expression. Briefly: *Agrobacterium* suspension containing P35S-35S-30B vectors was placed into a 5 ml Eppendorf tube, and then the root part of each

plant was placed into the induction medium (1x MS salt, 10 mM MES, 200 μM acetosyringone, 2% sucrose [MMA]) in the tube and cultured at 25±3 C° in a growth chamber under 16h light/8h dark cycles. Compared with the leaf infiltration technique, this new method is a simple and convenient process, and ease to scale-up. Moreover, the expression level obtained by root absorption was higher than the leaf infiltration method. It is assumed that the root absorption system may facilitate a rapid, large-scale and high expression level manner for the production of recombinant proteins in plants in the future.

Air-brushing

A further transient expression system for large-scale production of recombinant protein in plants is based on "air-brushing" an *Agrobacterium* suspension. In a paper published in 2015 Yang and his colleagues described a more convenient system than root absorption for the transient expression of foreign proteins in plants. The *Nicotiana benthamiana* leaves were agro-inoculated with an *Agrobacterium* suspension. The *Agrobacterium*-suspension was placed into an airbrush tube and then sprayed onto the leaves using the airbrush method. Whitman et al. (1999) at a 75-80 psi export pressure during spraying. The inoculated plants were covered with black plastic bags for 24 h for recovery. Next, they were cultured at 25±3°C in a growth chamber under 16h light/8h dark cycles. The proposed system is advantageous because it presents a fast, simple work procedure. For instance, when the *Agrobacterium* suspension is prepared beforehand, it only takes 20 minutes to spray hundreds of plants with suspension. Therefore, it is clear that when a large numbers of plants need to be agro-inoculated, the new system permits more advantageous and applicable for transiently expressing extraneous proteins (Jin et al., 2015).

Vacuum infiltration

Vacuum infiltration of *Agrobacterium* for transient gene expression in whole plant leaves is a fast, scalable, and useful approach for the production of foreign proteins with no need to generate transgenic plants (Fisher et al., 1999). During vacuum agro-infiltration, plants are set upside down and aerial parts submerged in *Agrobacterium* suspension. Then vacuum is applied causing gases to evacuate from leaf intercellular spaces through stomata. Rapid re-pressurization following

release of the vacuum results in the infusion of the *Agrobacterium* suspension into the leaf. Following vacuum infiltration of *Agrobacterium*, plants are further cultivated and target expression is monitored. The highest levels of target expression are typically observed 2-3 days' post-infiltration (dpi) with a binary vector and 4-7 dpi with a launch vector, after which the expression level typically decreases (Musiychuk et al., 2007). *Agrobacterium tumefaciens* is the most widely used vehicle for delivering a gene of interest into a plant for protein production. Agro-infiltration works extremely well in *N. benthamiana* but relatively poorly in most other plants, including *Arabidopsis thaliana.*

1.4-New advances in crop genetic engineering tools

The stable and gradual improvement in molecular biology has led to the advancement of new technologies to engineering plants. Traditional methods of crop transformation like *Agrobacterium*-mediated and particle bombardment are dependent on the random insertion of multiple copy of transgene into plant genome that may lead to gene silencing and unpredictable expression pattern. Because these restrictions, screening of stable transgenic lines with the desired level of transgene expression is labor intensive, slow and expensive (Ow, 2005). Many researches had focused on the deletion of random DNA integration and the reduction of the frequency of multi-copy transgene insertions and overll on techniques with a graeter consumer acceptance.

Recombinase technology

Site-specific recombinase systems were discovered in bacteria, yeast, and found to facilitate a number of biological functions. Site-specific recombination occurs at a specific sequence or recognition site and involves cleavage and reunion leading to integration, deletion or inversion of a DNA fragment without the gain or loss of nucleotides. Site-specific recombination (SSR) is a promising technology that can help to bypass most difficulties in genetic engineering. Since the 90s, three SSR systems have been described: the *Cre*-lox from *Escherichia coli* bacteriophage P1, the FLP-FRT from *S. cerevisiae*, and the R-RS from *Zygosaccharomyces rouxi.* This technology is reasonable to produce marker-free transgenic crop plants; but it may not be ideal due to constraints on time, labor and the substantial financial resources needed. Generation of transgenic crops in

this way necessitates multiple generations and may not be practical in species with longer generation times such as trees, or in other crops such as potato, which are propagated asexually. However, in 2006 Monsanto developed a first commercial marker-free corn LY038 based on *Cre*-lox system (Ow, 2007), which consists in a high content of lysine used as feed for poultry and swine. Recombinase-mediated excision has also been used in numerous crop plants such as wheat (Srivastava et al., 1999) and rice (Chawla et al., 2006) to determine complex insertion sites, containing multiple transgenes, down to a single copy. This also decreases the effort needed to screen the total number of transgenic plants to find a suitably expressing single copy line that is heritable. Rates of integration have been documented from ~33% in tobacco (Albert et al., 1995; Day et al., 2000) to almost 50% in rice (Srivastava et al., 2004).

Cisgenesis/intragenesis

The term "cisgenic plant" has been introduced a few years ago and it refers to "a crop plant that has been genetically changed with one or more genes" (containing introns and flanking regions like native promoter and terminator regions) selected from a crossable donor plant (Schouten et al., 2006a). It denotes that the genetically modified cisgenic crop encompasses genes maintaining their natural genetic composition, *i.e.* a perfect complete copy of a natural gene with all its regulatory elements. The font of a cisgene is the same plant species or a sexually compatible species as used for traditional breeding. However, contrasting traditional breeding, cisgenic crops contain solely the gene or genes of interest and no undesired genetic elements (Kamthan et al., 2016).

On the contrary, intragenesis indicates GMOs where the introduced gene originates from the identical species or a crossable species, but in contrast to cisgenes, intra-genes are hybrid genes, which can have genetic elements from diverse genes and loci (Rommens et al., 2007). As a result, the expression of a certain gene could be modified by using a different promoter or terminator regions.

The scientific community has interest in promoting less stringent regulations for cisgenic/intragenic crops. For instance, the European Commission (EC) requested the European Food Safety Authority (EFSA) to determine the hazards

of cisgenic/ intragenic crops compared to transgenesis or traditional breeding (EFSA Panel on Genetically Modified Organisms, 2012). Up to date, numerous traits have been incorporated into relevant crops by cisgenic or intragenic approaches. These species comprise potato (de Vetten et al., 2003; Rommens et al., 2005; Rommens et al., 2006; Rommens et al., 2008; Haverkort et al., 2009), apple (Joshi et al., 2011; Vanblaere et al., 2011), strawberry (Schaart et al., 2004), *alfa-alfa* (Weeks et al., 2008), perennial ryegrass (Bajaj et al., 2008), poplar (Han et al., 2011), barley (Holme et al., 2012) and durum wheat (Gadaleta et al., 2008). Recently, both intragenesis and transgenesis have been applied to different cultivated crops. Intragenic potato has been developed to produce high amylopectin content (De Vetten et al., 2003). An intragenic method also aimed to produce scab resistance in apples. Modification of tree architecture and growth rates is a major issue for the wood plant business. Based on the latter, a cisgenic method developed in poplar seeds to address both issues (Han et al., 2011). Until now, application of cisgenesis and intragenesis as alternatives to conventional transgenesis is limited to a few species, mainly because of the lack of knowledge of the regulatory sequences required. For several relevant crops, the current knowledge of full genomic sequences has opened up new possibilities to use genes and their native regulatory sequences to improve important traits (Espinoza et al., 2013).

Genome engineering/editing.

One of the newest approaches in engineered plants is genome editing. This method could be used first to determine the desired modification in plants and then to introduce chosen genetic variation in a rapid and accurate manner. The idea is to introduce targeted double-strand breaks (DSB) at a desired locus, which can be efficiently used to modify the genome of plants. Targeted DSBs can be realized using sequence-specific nucleases (SSNs) that are capable to recognize and split the selected locus with high specificity. What concerns the latter, four main classes of enzymes can lead to genome engineering: engineered homing endonucleases or mega-nucleases, zinc-finger nucleases (Kim et al., 1996), transcription activator-like effector nucleases (Christian et al., 2010), and clustered regularly interspersed short palindromic repeats (CRISPR)/Cas9 reagents. The latest addition to the family of sequence specific nucleases is the

CRISPR/Cas, which is proving to be the nuclease-of-choice for plant genome engineering. The innate defense system of prokaryotes involving CRISPR and Cas9 against an invading DNA element such as a virus or plasmid has been engeneered to versatile plug and play tool for genetic engineering.

Approaches	Examples	Advantages	Disadvantages
Conventional breeding			
Stable transfection	*Agrobacterium tumefaciens* Biobalistic Plastids Plants-cell suspension	Efficient, effective to improve many traits.	Inheritance of non-desired traits Time cosuming Random insertion of exogenous DNA
Transient expression		More targeted and rapid Introducing a transgene from an organism to host organism that cannot be crossed	Biosafety issues, environment and health hazards
Cisgenesis/ intragenesis		Greater consumer acceptance Environmental friendly	Require efforts to look for compatible gene pool
Recombinase technology	Cre-lox FLP-FRT R-RS	Site specific integration of transgene Merker-free plants	Not suitable for all species Screening of transformed plants is laborious and time consuming
Genome editing	Meganucleases Zinc finger TALEN /TALE CRISP/Cas9	Targeted and precise Engineering complex traits Useful for gene stacking	Focused to knockout gene

Table 5: Summarizes various approach than can be applied to generate genetically improved crop varieties and can be used in molecular farming (Kamthan et al., 2016).

1.5-Plastid transformation

Plastids are a group of organelles existing in the cells of plants, algae, and several protists and are crucial for viability (Waters and Pyke, 2005). The plastid genome provides an alternative site for the stable insertion of transgenes in plants and is frequently considered an 'environmentally friendly' form of plant genetic engineering, as plastid DNA (in most crops) is largely omitted from pollen. Chloroplast transformation was first proved for the unicellular green alga *Chlamydomonas reinhardtii* (Boynton et al., 1988) followed by the higher plants tobacco (Svab et al., 1990*a*) and tomato (Ruf et al., 2001). Since then, the chloroplast genome has been manipulated to produce vaccine antigens, commercial enzymes, hormones, antibodies, pharmaceuticals, and biomaterials, and engineered to deliver different agronomic traits, including resistance against various biotic (viral, fungal, and bacterial diseases) and abiotic (*e.g.* salt, drought, heavy metals) factors (Table 6). However, the number of crop plants in which chloroplast transformation has been reported remains very low and the technology has not yet reached success in the field.

Enzymes/protein	Gene	Host plants	Expression (%TSP)	References
Biopharmaceuticals				
A 1-antitrypsin	SERPINA1	Tobacco	2	Nadai et al., 2009.
Aprotinin	APR	Tobacco	0.5	Tissot et al., 2008.
Coagulation factor IX	CTB-FIX	Tobacco	3.8	Verma et al., 2010b.
Human serum albumin	Has	Tobacco	11	Fernandez et al., 2003.
Human somatotropin	hST	Tobacco	7	Staub et al., 2000.
IFN-γ	GUS-IFN-g	Tobacco	6	Leelavathi et al., 2003.
Enzymes/biomaterial				
Cellulases	Bgl1-,cel6B	Tobacco	5-40	Petersen and Bock 2011.
Trp	Asa2	Tobacco	ND	Zhang et al., 2001.
B-glucosidase	Bgl1	Tobacco	ND	Jin et al., 2011.
Pectin lyase	PecA	Tobacco	ND	Espinoza-Sanchez, 2015
Bacterial				
Anthrax antigen	pa	Tobacco	2.5-4.	Gorantala et al., 2014.
PWD	FaeG	Tobacco	1% DW	Kolotilin et al., 2012.
Viral				
Human papilloma virus	GUS E7	Tobacco	3-4	Morgenfeld et al., 2014
HIV	C4v3	Tobacco	25mg FW	Rubio-Infante et al., 2012
Rotavirus	Vp6	Tobacco	15 % TLP	Inka-Borchers et al., 2012
Agronomic traits				
Herbicide resistance	aroA bar			
Abiotic stress tolerance	g-TNT		7.7% TLP	Lin and Daniell., 2014
TLP: total leaf proteins; TSP: total soluble proteins; ND: not determined; DW: dry weight; FW: fresh weight (all concentrations in w/w).				

Table 6: Enzymes, biomaterials, vaccine antigens, and agronomic traits engineered via transformation of the higher plant chloroplast genome (*modified* from Ahmad et al., 2016).

This feature offers possibility to use chloroplast transformation to introduce natural and artificial operons into the plastome in a single transformation step. Nevertheless, post-transcriptional processes inside the chloroplast, such as RNA editing, removal of introns, processing of mRNA ends, and cleavage of larger mRNA transcripts (reviewed by Bock, 2015) should be taken into account. In addition, there are several factors limiting the technology.

Routine chloroplast transformation is currently limited to dicotyledonous plants and mainly members of the *Solanaceae* family, even if transformation of several crop plants has been successfully demonstrated, it is still not available for monocotyledonous plants except for rice (Lee et al., 2006).

Another important limitation is that expression of transgenes in non-green plastids is not as efficient as in green plastids (chloroplasts). Although the plastome is quite small, its contribution to the protein expression in photosynthetically active tissues can reach up to 50% of the total leaf protein content. In contrast, expression levels are considerably lower in non-green plastids (Zhang et al., 2012), which can result in a lower expression of foreign proteins.

Transgene containment in the chloroplast genome is not total. The desirability to use plants as 'green factories' lies in their scalability. Although plastids generally provide a degree of natural gene containment due to their maternal mode of inheritance, this containment is not absolute, as mentioned before.

Absence of glycosylation: plastids are capable of carrying out many of the post-translational modifications (PTMs) indispensable for the production of a physiologically active protein, such as the disulphide bond formation, lipidation, multimerization, and N-terminal methionine excision (Rigano et al., 2012). However, chloroplasts do not have the necessary machinery to carry out the glycosylation, very important PTMs in eukaryotes (Paul and Ma, 2013). The deficiency of glycosylation in chloroplasts may therefore be a bottleneck for the synthesis of those proteins that rely on this PTM for their correct functioning.

1.6-Optimization of the expression in host plants

Heterologous proteins are divided into three main groups: therapeutic proteins or those used for clinical diagnosis, proteins used as reagents for research and study commitments and proteins with different industrial purposes. Among these proteins, proteins used for therapeutic purposes constitute a singular class with the stringent quality standards and therefore require high value. A valuable expression system should (i) be capable of producing the needed protein with right conformation, (ii) have good productivity, (iii) be easy to handle and maintain, (iv) be safe and cheap, and (v) demand easy downstream processing. For the expression of required proteins in plant, DNA sequences can be integrated into the plants nuclear DNA through a suitable vector using appropriate transformation technologies. The gene should then be transcribed and translated. Ultimate expression of proteins is a quite complex process performed by many enzymes, co-factors, regulators, and compartmentalized in several sub-cellular organelles. In the last years, few steps have been identified as rate limiting which can be improved to increase expression of a desired transgene (Table 7).

1. Transformation and integration		
Attachment of SAR to DNA	Increased expression of GUS gene in tobacco cells up to 140-fold	Allen et al., 1996
2. Transcription		
Addition of *Myb* and leucine zipper	Increased expression	Boulikas et al., 1994 Hurst et al., 1994)
Hybrid promoter (Mac), of CaMV 35S and mannopine synthetase	*GUS* gene expression was increased 3–5 fold in leaves and 10–15 fold in hypocotyls and roots in tobacco and tomato plant, resp.	Comai et al., 1990
Addition of Intron 2 and 6 of maize alcohol dehydrogenase-1	Increase the expression of CAT (Chloramphenicol acetyl transferase) gene by 12-fold and 20-fold respectively in maize protoplasts	Mascarenhas et al., 1990
3. Translation		
Changing initiation codon context GGUUUAUGU to CCUCCAUGU	Increased expression	Gallie, 1993
Codon optimization in tobacco and tomato plants	Increase expression of cryIA (b) (*Bacillus thuringiensis*) up to 100-fold	Perlak et al., 1991
4. Final yield or protein accumulation		
Targeting to sub-cellular compartments e.g. apoplast	10^4-fold higher Expression of human epidermal growth factor (hEGF) was obtained in tobacco	Wirth et al., 2004

Table 7: Strategies used for enhancing expression of transgenes in plants.

Sometimes, a problem, which occurs in engineered plants, is the genetic expression level and the non-functional proteins production. This is generally observed with multiple copy integration at one or more sites, different base composition between foreign DNA and the integration site, detrimental effects of sequences adjacent to the foreign DNA integration site, and over expression effects.

1.6.1-Optimization by Codon Usage

The redundancy of the genetic code means that most amino acids are encoded by multiple synonymous codons. In all domains of life, a biased frequency of synonymous codons is observed at a genomic level, in functionally related genes (*e.g.* in operons), and within single genes. Although translation initiation is a fundamental step in protein synthesis, it is generally accepted that codon bias contributes to translation efficiency by tuning the elongation rate of the process. It is generally known that the genes in bacteria, mammals or humans are not very active in crop plants, in molecular farming become pivotal adjusting the DNA code to suit the "codon bias" typical of the crop plant species into which genes

from bacteria or mammals are introduced. Complete codon engineering by replacing codons with more favorable codons through the whole gene could generate partially or completely modified synthetic genes, which can improve the expression levels of the transgene (Sutton et al., 1992). This approach improved the expression levels of the transgenes "winter flounder antifreeze protein" (AFP) in corn (Georges et al., 1990), the porcine alpha lactalbumin gene in the seeds of transgenic maize and the B-subunit of heat labile enterotoxin of Enterotoxigenic *E. coli* (LT-B) (Lauterslager et al., 2001; Streatfield et al., 2002). Optimization of plant mRNA processing by replacing the AAT codon to GTG surrounding the translation initiation site of LT-B gene resulted in 5-40 fold higher quantity of LT-B expression level when transformed in potato (Mason et al., 1998). Different software programs are accessible for the optimization of codon usage, such as Gene Designer (Villalobos et al., 2006) and OPTIMIZER (Puigbò et al., 2007). Moreover, numerous strategies are used to inactivate the foreign DNA at an integration level: screening and selection for plants with single-copy integration, omitting repetitive homologous sequences and flanking exogenous DNA with the Scaffold Attachment Region (SAR). SARs are DNA sequences that stay specifically associated with the scaffold once the digestion of the chromatin with restriction enzymes occurs. SAR elements are common among different species and it is demonstrated that they are functionally preserved throughout evolution (Cockerill and Garrard, 1986a).

1.6.2 Synthetic biology

Recent progresses in genetic engineering made it possible to effect previously unobtainable genetic changes in most organisms, exposed to breeding, like plants. The genetic distance between the engineered organism and the source of the new genetic variation would be a functional measure for assessing the newness of the presented genetic changes. A more accurate and explicit nomenclature based on the genetic distance associated with the introduced genetic modifications is illustrated in table 8.

In order to increase and maximize expression of exogenous proteins in engineered plants synthetic genes can be used (xenogenic modification) as explained in Nielsen et al. (2003).

Categories	Source of genetic modifications	Genetic variability via conventional breeding	Genetic distance
Intragenic	Whitin genome	Possible	LOW
Famigenic	Species in the same family	Possible	↓
Linegenic	Specied in the same lineage	Impossible	
Transgenic	Unrelated species	Impossible	
Xenogenic	Laboratory designed genes	Impossible	HIGH

Table 8. Proposed categories for organisms currently designated 'transgenic' or genetically modified (Nielsen et al., 2003).

The aim of synthetic biology is to design artificial biological systems for novel applications. From an engineering point of view, construction of biological systems of defined functionality in a hierarchical way is essential to this emerging field. Neilsen et al. in a review published in 2013 described cells and the use of regulatory networks to execute computational operations to answer to their environment. Reliably manipulating such networks would be precious for many applications in biotechnology; for example, in having genes being expressed only under certain circumstances or applying dynamic or temporal control of expression, building such synthetic regulatory circuits is still one of the most challenging tasks in genetic engineering and as a result, they have not found extensive applications (Nielsen et al., 2003). New gene elements have been identified, remodeled or designed, which are further merged to build artificial biologic systems with specific purposes. From an engineering point of view, the design of a biologic system requires the appropriate disintegration of the design task into gene elements and their basic combinations able to operate in forms of circuits, devices and modules, and then uses a bottom-up methodology to further assemble them into more complex systems in an hierarchical manner. Ten years after the first two synthetic elements were introduced in this field (Elowitz and Leibler, 2000; Garden et al., 2000), synthetic biologists have made advancements to engineer a huge assortment of artificial gene parts to enlarge their functionalities and applications. The goal of synthetic biology is to extent or change the behavior of an organism and engineer them to make them execute a new task. Recent developments in synthetic biology towards engineering complex living systems thought novel assemblies of biological molecules. Recent advances have demonstrated successes in the design of some genetic circuits, devices and modules, especially those for the regulation of the genes expression. However, these designs are predominantly

for proof of concepts and far from generalization and rationalization. A more focused and targeted attempt on the design parts list could ease the standardization of design parts. Future work could be focused on systematically mapping functional motifs and calculating the effects of their combinations as linear signal cascade and in the framework of complex connections (Zhang et al., 2010).

1.6.3-Optimization of Promoters

The upstream element of a gene called promoter is an essential part in the gene expression, because of the presence of sites with a specific core sequence to which RNA polymerase and transcription factors bind. By using different promoters, the regulation of protein synthesis in plants can be improved. Table 9 shows several promoters used in molecular farming.

Promoters	Origin	Type of promoter	Reference
CaMV 35S	Virus	Constitutive	Odell et al., 1985
C1	Cotton leaf curl multan virus	Constitutive	Xie et al., 2004
Polyubiquitin-1	Maize	Constitutive	Christensen et al., 1992
Actin	Rice	Constitutive	An et al., 1996
Pristinamycin-responsive	Virus	Inducible	Frey et al., 2001
In2-2	Maize	Inducible	De Veylder et al., 1997
Nopaline synthase	Agrobacteria	Inducible	Shaw et al., 1984
Mannopine synthase	Agrobacteria	Inducible	Langridge et al., 1989
Patatin	Potato	Tuber specific	Liu et al., 1991
GBSS	Potato	Tuber specific	Visser et al., 1991
Maize globulin-1	Maize	Embryo specific	Belanger & Kriz, 1991
B-Conglycinin	Soybean	Embryo specific	Chen et al., 1986

Table 9: Most frequently used promoters in plant transformation (Desai et al., 2010).

The promoter is a genetic element that regulates when and where the transcription occurs. Generally, the promoters are constitutive, inducible and specific for a certain tissue. Based on these issues is it possible to maximize the expression levels of a certain protein. Constitutive promoters deliver high expression levels. The most common used constitute promoters are the cauliflower mosaic virus 35S (CaMV 35S) promoter and the maize ubiquitin-1 (used in dicots and monocots, respectively) promoter. These promoters have a viral origin and deliver an increase in the expression of a selected gene. However, also some harmful effects such as gene silencing *via* co-suppression have been reported (Sutton et al., 1992). With the help of constitutive promoters,

extensive expression of foreign genes in plant cells can be obtained and this could lead to an amassing of recombinant proteins in the cells, which at the end leads to be toxic for the cells. This problem can be skipped by employing inducible or tissue specific promoters (Majumadar, 2013).

Wound inducible defiance gene originating promoters are known to be used for the expression of recombinant proteins in plants (Cramer et al., 1996; Hansen et al., 1997). In plants tissue specific promoters can be used and to target the expression in certain tissues that have a lower metabolic activity such as seed embryos or endosperm. This is an advantage in the downstream purification process. The maize globulin-1 promoter used to target the protein expression to the embryo of monocots (Belanger et al., 1989) is a popular tissue specific expression promoter. Other tissue specific promoters like the sps1 promoter for leaf-specific expression (Chavez-Barcenas et al., 2000), the patatin promoter for tuber-specific expression (Park et al., 2002), are well known. There are several other reported seed specific or embryo specific promoters (Majumadar, 2013). In particular, in the case of the development of an oral vaccine where edible parts of plants are directly used for the immunization, tissue specific promoters represent the main choice. The expression levels in the seeds of monocots using seed-specific promoters are higher than those obtained when using constitutive promoters.

1.6.4-RNA processing, intron mediated enhancement and stability

RNA processing steps such as capping, splicing and poly-adenylation can have an impact on the expression levels of therapeutic proteins. Sequences downstream to stop the codon are critical for the processing and should incorporate signals targeting the message for poly-adenylation. Poly-adenylation sites also influence the solidity of the message and the protein expression levels in plants (Lin et al., 2009). After the transcript is being synthesized, the RNA stability is important to achieve a high expression level. There are specific recognition sites that reduce the RNA's half-life (Sullivan and Green, 1993). Some of these sites are known and probably correspond to specific binding sites. To increase the accumulation of recombinant proteins, it could be necessary to identify, modify or delete them.

1.7-Targeting protein

As explained before, it is possible to target the expression of proteins in a specific organelle or specific part of the plant. Protein targeting is important and should be optimized to get a better output. After translation, proteins are folded and then moved to intercellular locations through molecular chaperones, which protect the native protein from degradation. Protein targeting is needed for its stability, modification and for the survival of the plant. Proteins secreted in cytosol are often degraded by proteases and until today protease free plant lines are is still not possible to establish. Glycosylation and disulfide bridge formation in the recombinant protein is only possible in the endoplasmic reticulum (ER) and in the Golgi apparatus of plant cells. Therefore, after translation, the glycoproteins need to be transported to ER or Golgi apparatus (Iturriaga et al., 1989; Horvath et al., 2000). Targeting proteins to intracellular organelles (such as the ER, the chloroplast, and the vacuole) or secretory pathway (like the ER or the Golgi apparatus) eases the proper folding of proteins, which is crucial for their appropriate functioning and increased expression levels (Pelham et al., 1990; Schouten et al., 1996). By incorporating C-terminal ER retention signal, 10-100 fold increases in the target protein yield is achievable as when targeting them to the secretary pathway (Conrad and Fiedler, 1998). Improved accumulation of LT-B in tobacco and potato has been observed by directing the recombinant protein to the microsomes (Mason et al., 1995). As mentioned before, targeting the production of proteins in endoplasmic reticulum is more convenient compared to the cytosol. Certain ER retention signal sequences like the KDEL and the HDEL are used to minimize eventual protein degradation, since the ER comprises very small amounts of proteases and offers a quite protective environment.

1.8-Seed based platforms

Recently, most pharmaceutical proteins are produced in leafy crops for obtaining great biomasses. However, leaf proteins during harvest are easier degraded with proteolytic enzymes in plants. In addition, long-term storages of leaf material are also tricky. Based on preceding experiences, overexpression of foreign proteins in leaf cells can also result in necrosis and cell death (Phoolcharoen et

al., 2011). Seed-based systems have extra advantages because they exploit the natural storage properties of seeds to ease batch processing and distribution. The stabilizing effect of seeds after harvest permits the recombinant subunit vaccines and antibodies to be transported via the mucosal route, as they are more able to survive the harsh environment when protected by the plants matrix. Seeds have proven to be adaptable hosts for recombinant proteins of all types, including peptides or short and long polypeptides as well as complex, noncontiguous proteins such as antibodies and other immunoglobulins. The extraction and recovery of recombinant proteins from seeds is greatly assisted by their dormancy properties, since this allows a long-term stability of stored products including recombinant proteins and a separation of the processing from the growth and harvest cycles. Furthermore, the low water content and relatively low bio load of seeds help in designing relatively cheap manufacturing processes for the desired active pharmaceutical ingredient (Boothe et al., 2010). Seeds offer themselves to many applications in the area of molecular farming. A vast number of species have been investigated including A*rabidopsis*, barley, *Brassica* spp, corn (Streatfeld et al., 2002), pea (Perrin et al., 2000), rice (Szarka et al., 2006) and soya bean (Philip et al., 2001). In rice for example, the use of storage protein promoters to coordinate the expression of genes for mammalian proteins such as human lysozyme, resulted in an average expression level of 13%–14% of the total soluble protein (Huang, 2004), which is quite good within an economic threshold for the target protein. Antibodies and scFv proteins in a variety of seed production hosts accumulate at levels of about 1%–5% of the total seed protein (Fiedler et al., 1997). The use of seeds for the recovery of a recombinant protein is advantageous also in other ways as the scale of production increases. Firstly, the intrinsic bioburden on seeds is lower than in vegetative structures such as leaves. Although neither seed nor leaf extraction can be performed at a large scale under aseptic conditions, most seeds can be subjected to a surface 'sterilization' technique, which reduces bio-load to industrially accepted standards for a raw material. A further example described by Virdi et al. (2013), is about anti-$F4^+$ ETEC antibodies in seeds of *Arabidopsis thaliana*, whose transformation is easier compared to that of feed crops, and sufficient antibody-producing seeds can be up-scaled in greenhouses in a quite short time. The seeds provide an antibody production platform with ease of

storage at high concentrations in a confined space, and convenience of oral administration, which is considerably advantageous for large herds of piglets (Lau et al., 2009). More importantly, the crushed seed matrix could protect the antibodies from gastric digestion by outcompeting proteases, as proved in the study of in-pea-seed–produced anti-*Eimeria* antibodies administered in chicken fodder (Zimmermann et al., 2009).

1.9-Suitable host and model organism in Molecular Farming

1.9.1-Arabidopsis thaliana

Arabidopsis thaliana has been historically the foremost used plant for genetic transformation. Among the traits of *Arabidopsis*, there are some interesting characteristics that have led scientists to exploit this model plant such as: the short generation period, a small genome size, a presence of a self-pollination mating system, an ease of *in-vitro* culturing, easy regeneration and *in-vivo* transformation, as well as its lack of food and feed applications (Koornneef and Meinke, 2010). In particular, *Arabidopsis* is capable to produce significant yields of seeds over a short period. This ability makes it particularly suitable for the production of many compounds (such as nutraceuticals, therapeutic human proteins, vaccines, and antibodies). *Arabidopsis* should be transformed during its early growth stages, since its development towards the flowering phase could increase the risk of environmental contamination (Ruebelt et al., 2006). High levels of recombinant protein accumulation in *Arabidopsis* seeds have been achieved by employing a seed-specific expression cassette (Van Droogenbroeck et al., 2007). It is a non-food/feed crop for which a very efficient and easy *in-vivo* transformation method has been developed, known as floral dip (Clough and Bent, 1998). *A. thaliana*'s fast generation cycle in combination with prolific seed production turns it into a very adaptable production system. Therefore, it can be used for the quick production of stable homozygous lines (about nine months) and very fast production of diverse recombinant proteins.

1.9.2-Cereals and legumes

Cereals and legumes are extremely suitable for the production of a lot of useful protein compounds. Xue et al. (2003) investigated the use of barley as a bioreactor to produce a highly active and thermo-stable hybrid cellulase (1-4b–glucanase). Barley is also an important host for the production of valuable compounds like serum albumin, lactoferrin, lysozyme, human antitrhombin III (Sthal et al., 2002). Barley is suitable for the production of biopharmaceuticals. The recombinant single-chain Fv antibody has been produced in order to control carcinoembryonic antigen in wheat as well as in rice and in both crops the proteins could be conserved for up to 4 or 5 months at room temperature without any reduction in activity of the product (Stoger et al., 2000; Ma et al., 2003). The successful employment of cereal crops is due to the long-term storage of proteins whit an appropriate biochemical environment, a low water content and low protease activity. The lack of phenolic components is another advantage of cereals seeds compared to other plants, such as tobacco. Generally, the downstream process of depleting phenol from the products is a too long and expensive process (Ma et al., 2003). In addition, using cereals and legumes in molecular farming is disliked by "Public Opinion" because they are a valuable resource for feeding people and animals. The challenge described above appear both companies' business strategies and focus of innovation. Important changes identified include a shift away from major food crops.

Comparing MMF activities at present to a couple of years ago, reveals a noteworthy shift from food crops to non-food crops or minor food crops. As illustrated in table 10 the number of field trials with major food crops went down since 2003 in particular field maize which was considered to be the most valuable source for feeding in human and animals.

Plant	Number of applications for field trials	
	Up to 2003	Update from 2004 onwards
Maize	72	14
Soybean	12	0
Rice	8	18
Barley	1	0
Wheat	1	0

Table 10: Food crops species used for MMF (by Spok and Karner, 2008).

1.9.3-Vegetables and Fruits

Notably, fruits, vegetables are consumed in the form of moderately processed or entirely raw foods. These type of plant products are normally free of toxicants and are rich in nutrients, which make them particularly suitable to produce recombinant vaccine subunits, antibodies, and food additives for active immunotherapy (Ma et al., 2003). Potatoes have been frequently used because of the transformation protocols to generate transgenic lines, which are established. Tuber extracts from the transgenic lines expressing the S1 glycoprotein gene of infectious bronchitis virus (IBV) have been demonstrated to protect the chickens from clinical diseases, as well as virus shedding when challenged (Zhou et al., 2003). Potatoes have been used extensively for the production of plant-derived vaccines, which have been administered to humans in many clinical trials. Among several other antigens expressed in potatoes for human trials, the enterotoxigenic *E. coli* (ETEC) labile toxin B-subunit (LTB), one powerful oral immunogen (Tacket et al., 1998) and Norwalk virus capsid protein (NVCP) (Tacket et al., 2000) had noticeable success. A recent trial using LTB expressed in processed corn seeds produced quite similar results to those of the potato study (Tacket al al., 2004). Besides, the risk of outcrossing in the open field production is low as the plant can be clonally propagated. In addition, as the industrial processing of tuber is established, the cost of downstream processing can be reduced. However, potato tuber has a relatively low protein content (Mason et al., 2002) and it is not palatable although to be eaten raw. While cooking can improve its palatability, it might lead to the denaturation of the foreign protein, thus resulting in poor immunogenicity if it has to be used to produce vaccine antigens (Gunn et al., 2012). Another candidate crop are tomatoes. Tomatoes have become a more attractive alternative system, since they are palatable and can be eaten raw with no need to be cooked. Thus, vaccine antigens expressed in them do not risk to be denatured by heat treatments. The first vaccine candidate used for expression in tomatoes was the rabies virus glycoprotein (McGarvey et al., 1995). Furthermore, they had been used to express the capsid proteins VP2 and VP6 of rotavirus, which were immunogenic to mice by intraperitoneal delivery (Saldaña et al., 2006) The inherent high amounts of vitamin A in tomatoes could also help in amplifying the immune responses (Gunn et al., 2012). Tomatoes have an established industrial

cultivation and processing system just like potatoes. However, the fruits have also a quite low protein content and must be chilled after harvest in order to avoid spoilage. Although the freeze and drying technology is available to preserve the fruits, this adds an additional cost to the processing.

Among the fruits, banana is a significant one with many advantages. Bananas are consumed as staple food, are available at low cost in tropical and subtropical countries and are also palatable and easily digestible.

1.9.4-Nicotiana tabacum

Nicotiana tabacum is a largely used host system in MMF (as well as *Arabidopsis thaliana*). *Nicotiana tabacum* is a model organism and an extremely adaptable system for all the main aspects of cell and tissue culture researches. Tobacco is a natural allotetraploid generated through two hybrids between two diploid progenitors, *Nicotiana syslvestris* and N*icotiana tamentasiformis* which occurred approximately 6 million years ago (Okamura et al., 1985*).* Nonetheless, the application of tobacco in smoking, it has many unique advantages compared to other plant species for the production of pharmaceutically important proteins. Scientists generally think that the role of tobacco in recombinant-protein investigations is close to that of the white mouse in mammalian studies of the last 20 years. As a leafy species, tobacco has numerous advantages compared to other plants, which have encouraged researchers to focus on this plant as an undeniable alternative for recombinant protein expression. This herb can produce a biomass of up to 100 ton/ha and tobacco is a very prolific seed production of about 1 million seed/plant (Ganaphati et al., 2004*).* Moreover, it is a well-established system to transform tobacco that results in high levels of soluble protein. The potential of utilizing various strategies for the expression of proteins in a stable or transient manner using this species, as well the option to utilize chloroplast genome-based methods using *Agrobacterium* or viral induction are its other advantages (Karg and Kallio, 2009). Tobacco is not a feed or food crop, and this decreases the odds of its contamination within the feed or food chains. In addition, tobacco can produce a large number of therapeutic immune-modulatory molecules, like cytokines, vaccines and antibodies (Tremblay et al., 2010). The high concentrations of alkaloids and nicotine in some tobacco varieties are disadvantageous for the usage of this plant for

molecular farming. These last characteristics have been compensated by breeding new cultivars such as “81V” (Ma et al., 2003). In addition to avoid this problem, seed-based products in tobacco are developed instead of leafy products. In table 11, biopharmaceuticals products in tobacco plants are shown.

Type of protein	Name	Reference
Monoclonal antibodies	Anti-PA (Protective antigen)	
	Anti-BoNT/A scFv (botulinum neurotoxin/antibody single chain variable fragment) idiotype specific antibodies	
	Anti-Anthrax	Hull et al., 2005
	Cancer B-cell lymphoma	Young et al., 2009
	Breast and colon	Brodik et al., 2006
Cysteine proteases	Der p 1 (Dermatophagoides pteronyssinus allergen)	Johnston et al., 2009
	Der p 2	Lienard et al., 2007
Proteins	Ebstein-Barr virus	
	HIV p24 capsid protein	Zhang et al., 2002
	Tet-C Tetanus toxin fragment C	Tregoning et al., 2009
	UreB urease subunit beta	Gu and Glatz, 2007
Glycoprotrein	Interleukin 13	Thompson and Debinski, 1999
Growth hormone	Human somatotropin	Staub et al., 2001
Anticoagulant	Human protein C	Crammer et al., 2002

Table 11: Main biopharmaceutical products in tobacco plants.

The availability of various tobacco expression systems with their particular strengths is another advantage of this model plant over other plant species. Nuclear transformation is the appropriate method to achieve the long-term production of glycoproteins, such as antibodies. Alternatively, chloroplast-based expression systems allow the production of large amounts of proteins that require certain types of post-translational processing. Tobacco is amenable to several different simultaneous applications and has the potential to excel at each of them. When a product that requires rapid modification, as in the case of the abovementioned idiotypic anti-cancer vaccine, transient expression can be anticipated to produce the appropriate quantities of the chosen protein within a short period (Tremblay et al., 2010). By using transient protein-expression systems, researchers can produce significant amounts of recombinant protein during a short period, which is essential for the rapid response to a disease

outbreak, as is the situation when a new influenza appears, *e.g.*, during the A/H1N1 pandemic, as well as for patient-specific cancer cures.

1.10-Edible Vaccines

Vaccines are a biological preparation that provide active acquired immunity to a particular disease. A vaccine typically contains an agent that resembles a disease-causing microorganism and is often made of weakened (*i.e.* attenuated) or killed (*i.e.* inactivated) forms of the microbe, its toxins or one of its surface proteins. The agent stimulates the body's immune system to recognize the agent as a danger, dismantle it, and keep a memory of it so that the immune system can more easily recognize and destroy any of these microorganisms that it could encounter subsequently (Abbas et al., 2015). Vaccination has greatly lowered the burden of infectious diseases and has led to the eradication of smallpox, near eradication of polio, and the prevention of huge numbers of deaths (Plutkin et al., 2008). Traditional vaccines, attenuated or inactivated, present the risk of reversion into pathogenic forms, thus the production of these vaccines usually requires high-level biosafety facilities. For these reasons in the last two decades, subunit vaccines are developed and used widely. Subunit vaccines are composed by major antigen components of the disease-causing organism complexed with adjuvants. After the administration, subunit vaccines will provoke immune response in the host. Subunit vaccines can be given to animal or human with weakened immune systems. The potential advantages of using subunits as vaccines are the increased safety and less antigenic competition, ability to target the vaccines and differentiation of vaccinated animals from infected animals is easy. Since only a few components are included in the vaccine ability to target the vaccines at the site where immunity is required is possible. Potential disadvantages of subunit vaccines are that they generally require strong adjuvants with possible tissue reactions. The duration of immunity is generally shorter than the live vaccines.

Most currently available vaccines are delivered by injection, which makes mass immunization more costly and less safe. Oral vaccines, needle-free, have several attractive features compared with parenteral vaccines, in particular to protect against mucosal transmitted pathogens. Orally delivered vaccines are processed

and presented by the digestive tract's immune system, often referred to as the gut-associated lymphoid tissue (GALT). The GALT is a complex system consisting of inductive sites (where antigens are encountered and responses are initiated) and effector sites (where local immune responses occur) linked by a homing system, whereby cells induced by antigen in the GALT migrate to the circulation and, subsequently, colonize the mucosa. As a result, oral vaccination can induce immune responses locally in the gut and at distant mucosal sites, as well as systemic humoral and cellular immune responses. Oral vaccination typically generates a large amount of secretory IgA (sIgA), which plays a major role in mucosal defense. Accordingly, studies have focused on pathogens that enter the body through mucosal surfaces and cause diseases of the intestinal, respiratory and genital tracts.

Edible vaccines (oral vaccines) are transgenic plants expressing a virus or bacterial antigens (subunit vaccines), that can be administered raw with no need to be previously processed and act as a vaccine inducing a sufficiently protective mucosal immune response versus a particular disease (Dus Santos and Wigdorovitz, 2005). Several benefits are related to edible vaccines, *e.g.* they represent a suitable method of administration; moreover, the oral administration *via* food or feed, allows the activation of mucosal immune response where the major pathogens gain access to the body. Plant-based edible vaccines do not require a cold chain to preserve the vaccine; hence, the technology is very useful for vaccines to be used in countries without concrete health infrastructure (Third-World countries). Plants or plant products, which are edible, provide the additional unique opportunity to serve as vehicles for oral delivery of the vaccines.

During the past twenty years, plants drove the attention in vaccinology as both production hosts and delivery vehicles. Looking to minimize costs, the production of subunit vaccines becomes advantageous if a vegetable organism is used as the expression host for the recombinant vaccine and if its biomass can support a straightforward formulation of an oral vaccine. In terms of easy administration and immune protection at local mucosal tissues, oral vaccination is an attractive approach to fight against several diseases, particularly in poor countries where vaccines are more needed.

After 25 years of exploring the concept of plant-based vaccines, several edible plants have been used to develop plant-based vaccine candidates. The main attribute is that edible plant tissues are very cheap to produce in a process that can be easily scaled-up. Plant cells can be genetically engineered to transiently or stably express antigens in the nucleus or in the chloroplast. Nuclear expression offers high biosynthetic capacity because complex post-translational modifications take place. A myriad of proof-of-concept have been published in the last years. Several vaccine prototypes were thoroughly analyzed at the preclinical level targeting virus (Yusibov et al., 2002), bacteria (Lakshmi et al., 2013; Uvarova et al., 2013; Zhang et al., 2013), and parasite pathogens as well as autoimmune diseases (Ruhlman et al., 2007). Many preclinical studies against several diseases are going on with the aim to enter clinical trials or trials in definitive animal hosts for veterinary species (Yuki et al., 2013; Lindh et al., 2014, and Hayden et al., 2015). Remarkably, several clinical trials have been executed to verify the safety and immunogenicity of oral plant-based vaccines including antigens from HBV, delivered in potato (Thanavala et al., 2005); enterotoxigenic *E. coli*, delivered in maize (Tacket et al., 2004), Newcastle disease virus, in potato (Tacket et al., 2000); and rabies virus, in spinach. The findings reflect a potential to induce specific humoral responses in humans. The first plant-produced biopharmaceutical approved for human use (taliglucerase alfa for Gaucher's disease treatment, named ELELYSO®) was produced in carrot, and this species provides an excellent platform because of the experience gained in terms of production at the industrial level and, moreover, the establishment of proper conditions that meet the regulations governing biopharmaceuticals for human use.

1.11-Challenges of Plant-Based Vaccines

Although many plant-based vaccines that have been created are still in phase 1 clinical trials, some vaccines have advanced or completed phases II and III trials (Mardanova et al., 2015). Nevertheless, until today, there is no plant made vaccine that has been approved to be marketed for human or animal consumption. Thus, it is worthwhile to note that even though the production of plant-based vaccines had been initiated almost two decades since 1989 (Almada et al., 2015), a few challenges still have to be achieved in order to develop them

into highly efficient vaccines. The issues that need to be addressed could start from the upstream processes to the implementation of the vaccines. Generally, the three main challenges are: the selection of the antigen and the plant expression host, the consistency of the dosage, and the manufacturing of vaccines according to Good Manufacturing Practice (GMP) procedures.

1.11.1-Selection of Antigen and Plant Expression Host

The first issue is the selection of an antigen and the right plant expression host. This stage is very important in developing a vaccine that is able to fulfill all the requirements needed because not all antigens are well-matched with the selected host plants (Arnold et al., 2012). The correct and careful selection, will not only help to determine the safeness of the vaccine produced, it can also be utilized to produce thermal-stable vaccine (Arnold et al., 2012). Meanwhile, the identification of antigen candidates of poorly characterized pathogens with promising characteristics can be done by applying genomics or proteomics approaches (Bader et al., 2012).

1.11.2-Consistency of Dosage

The consistency of dosage is another challenge that the researchers have to face as dosage produced may vary within the plants of the same species, from fruit to fruit and from generation to generation due to the size and ripeness of the fruits or plants. The transgenic plants show intrinsic variability in the antigen expression due to the position and pleiotropic effects caused by generic integration of the transgene within the host plants genome (Foged, 2012). On top of that, it is also quite difficult to evaluate the required dosage for every patient. Levels of innate and adaptive immune responses generated in different individuals may vary based on the types of antigens being exposed in the body. Between two patients with different body weight as well as their age, the dosage of plant-based vaccine required will be different. If this issue is not well - monitored, an immunological tolerance will be induced when the patient is overdosed while reduction in antibody production will occur when the patient is under dosed (Arnold et al., 2012). Besides that, gene silencing might be induced due to the increase of mRNA in the transgenic plant cells as the growth of the plants is stopped and the fruit formation is reduced whereas the antigen content

is increased (Berhate et al., 2014). In such cases, consumption of plant-based vaccines may induce allergic reactions and few side effects such as toxicity on central nervous system, cytokine-induced sickness, and autoimmune diseases.

1.11.3- Manufacturing of Vaccines according to GMP Procedures

The ultimate goal of plant-based vaccines is to produce stable transgenic vaccines, which are safe for consumption while reducing the production cost. Besides all the underlying issues, that may affect the efficacy of plant-based vaccines, the manufacturers shall ensure their responsibility to follow the Good Agricultural Practices (GAP) and Good Manufacturing Practice (GMP) so that the upstream to downstream production of plant-based vaccines is strictly controlled for quality management. Generally, to produce a plant product that could meet the quality standard, the bio manufacturing facilities must be well equipped so that complete processing cycles of the plant vaccines could be accomplished. The facilities include equipment for plant and bacterium cultivation, infiltration, plant harvest, and protein purification (Ma et al., 2014). Takeyama et al. (2016) also summarized a few GMP plants that produce various vaccines such as influenza HA antigen, Norovirus capsid protein subunit vaccine, and rice-based cholera vaccine (Ma et al., 2014). Concurrently, Kashima et al. (2016) reported that in order to produce a plant vaccine that meets the governmental regulatory requirements, many steps and precautions need to be taken into consideration. During the production of a rice-based oral cholera vaccine, MucoRice-CTB, the biomanufacturing agency successfully established specific techniques to maintain the seed of MucoRice-CTB. The agency further evaluated the seed’s propagation and stored seeds were renewed periodically to maintain the good quality. Furthermore, cultivation of the plant using a closed hydroponic system helps to minimize the variations in vaccine production. The rice produced was polished, powdered, and packaged to make the MucoRice-CTB drug substance. A final control on the identity, the potency, and the safety of the MucoRice-CTB product must be executed and only the products that satisfy the quality requirements will be released (Su et al., 2015). It remains a great challenge to maintain the GMP standard for the product in plant-based vaccine industry. Besides the equipment, facilities, and method used to produce the vaccine, there are other considerations that have to be taken into account like

those stated in the GMP guideline published by WHO (Govea Alonso et al., 2014; Valdez et al., 2014). GMP for biological products guideline stated that some particular precautions are necessary for the manufacture, control, and administration of biological products as procedures and processes used in the production usually lead to high variation in the quality of products. Thus, the precautious steps should start from the very beginning of the production processes. However, in-process control is also important during the manufacturing of the biological products. Skillful staff is required to run the production processes and thus the bio manufacturing agency should provide necessary training to the staff. Buildings for the vaccine production must be designed in a way that operations can be carried out smoothly. A special design is required for plant vaccine production, in which the seed lots should be stored separately from other materials. Some other general rules of GMP shall be followed to maintain the quality standard of the vaccine products. These include the facts that standard operating procedures shall be implemented for all manufacturing operations, all products shall be clearly labelled, lot processing and distribution records shall be properly kept, and quality assurance and control shall be in place in monitoring the product quality.

Many medicinal products including edible vaccines are derived from proteins, *e.g*. insulin, vaccines and antibodies and has been extensively studied, as application of Molecular Farming, due to the high value of these recombinant proteins. In Europe, legislation (Council Directives 2001/82/EC and 2001/83/EC) defines a medicinal product for both human and veterinary use as *"Any substance or combination of substances presented for treating or preventing disease. Any substance or combination of substances which may be administered to human beings with a view to making a medical diagnosis or to restoring, correcting or modifying physiological functions in human beings is likewise considered a medicinal product*". All medicinal products (with or without genetic modifications) have to be tested thoroughly prior to commercialization.

a. Clinical trials for human use

After mandatory preclinical research and animal trials, there are three clinical phases for experiments on human subjects. In a first phase, the safety of a

medicinal product is tested on a small group of healthy people. In the next phase, the optimal dose is defined for a group of patients. Finally, in a third clinical phase the activity of the medicinal product is tested. All tests from the preclinical research until commercialization of a new pharmaceutical can easily last from 10 until 12 years. Directive 2001/20/EC regulates clinical trials with medicinal products for human use. At the start of a clinical trial, the subject has to provide his free and informed written permission, and an ethical commission has to give a positive advice. In addition, the minister of public health has to give his written permission for clinical trials with medicinal products containing GMOs. The clinical trial also has to comply with Council Directives 2009/14/EC and/or 2001/18/EC, respectively for contained use and/or deliberate release into the environment. At the European level, it is not clear yet when a clinical trial is part of contained use, but for now all clinical trials performed in the hospital itself are subjected to Council Directive 2009/14/EC concerning the contained use of GMOs.

b. Clinical trials for animal's use

There is no separate legislation for clinical trials for veterinary use, but they have to comply with chapter II of the RD of 21 February 2005 concerning the deliberate release of GMOs into the environment. The only difference is that the competent authority taking the decision is the Federal Agency for medicines and health products. In addition, permission from the ethical commission is needed before initiating the trial.

c. Commercialization

Regulation (EC) no 726/2004 regulates the procedures for permits and supervision of medicinal products for human and veterinary use. A medicinal product made with recombinant DNA technology or controlled expression of genes for biologically active proteins in prokaryotes and eukaryotes can only be commercialized when a permit of the Community is granted. The permit will be valid in all member states of the EU. The procedures for medicinal products for human and veterinary use are quite similar. A permit request is applied at and evaluated by the European Medicines Agency (EMEA). If the advice is positive,

the dossier is provided to the European Commission, which can grant permission. This permission is valid for five years. Afterwards, the permission can be extended after an evaluation of advantages and risks by EMEA. This extended permit is unlimitedly valid. To obtain permission for the use of medicinal products for veterinary use, which will be used for food, some specific regulations need to be applied (Regulation EC no 2377/90).

1.12-Edible Vaccines in Veterinary Medicine

Recently, the World Health Organization, the UK government and the G8 governments have emphasized the need for judicious use of antibiotics in agriculture as a key element of strategies to prevent or delay the onset of antibiotic resistance (G8 Science Ministers Statement, 2013, UK Department of Health, 2013 and World Health Organization, 2016). These initiatives, coupled with a growing public demand for animal-based food, which is "produced without antibiotics", will undoubtedly constrain the availability and routine practice of using antibiotics for growth promotion and prophylaxis in livestock, poultry and fish production. Within this context, it is imperative to devise cost-effective strategies for the intensive production of livestock and fish using fewer antibiotics. Increased use of vaccines and immunotherapeutic agents will be a cornerstone of these strategies (Topp et al., 2016). Vaccination of animals against an infectious disease is a control method that has been practiced for over a century with remarkable success. Prior to the past two decades, most veterinary vaccines were either inactivated products formulated with an oil-based adjuvant or live attenuated vaccines. The field of biotechnology and molecular immunology yielded rapid advancements starting in the 1980s, including the ability to produce subunit antigens in a cost effective fashion for the veterinary market. However, protection of animals against infection remains a major challenge in most of the developing countries, especially in the surge of drug resistant strains. Vaccine targets are also changing, with non-infectious disease targets representing a considerable growth area. For example, these include molecules to control fertility, behavior and production by immunization against hormones or hormone receptors. In addition, protein-misfolding targets such as prion diseases are of significant interest for the control of Bovine Spongiform Encephalopathy (BSE), Chronic Wasting Disease (CWD) and scrapie, largely as

a means of mitigating the threat of transmission to humans or trade barriers (Gerdtsa et al., 2013).

As mentioned before edible vaccines are plants, or part of them, engineered for the expression of antigens. After the oral administration, they induce a protective mucosal immune response. Oral delivery offers ease of application and protection against pathogens interacting with host mucosal surfaces *via* the induction of mucosal immunity (Streatfield et al., 2005). Plant-based expression systems represent an interesting production platform due to their reduced manufacturing costs and high scalability (Clemente et al., 2012). The cost for vaccine injections in animal livestock is related closely to the productivity of the industry, plant-based edible vaccines represent a viable alternative from the perspective of production cost (Virdi and Depicker, 2013). The obtained recombinant antigens retain the same structural integrity and activity. These transgenic plants safely and effectively deliver non-replicative subunit vaccines through the consumption of edible parts (Hefferon, 2010).

Mucosal surface, which covers the digestive, respiratory and reproductive tract, is the largest immunologically active tissue in the body (Tacket et al., 1999). Edible vaccines can stimulate both mucosal and humoral immunity.

The antigens in transgenic plants (in particular in seeds), are delivered through bio-encapsulation, *i.e.,* the tough outer wall of plant cells, which protects them from gastric secretions and finally break up in the intestine. The released antigens are then taken up by M cells, present over Peyer's patches and over gut associated lymphoid tissue (GALT). These antigens are subsequently passed onto macrophages, other antigen presenting cells and local lymphocytes. This triggers formation of specific serum IgG, IgE and local IgA antibodies and memory cells. These antibodies can neutralize the target molecule of the parasite on subsequent exposures (Lal et al., 2007). So many trials have been conducted to express either target antigen or antibody in edible part of the plants. These can be fed to animals directly without any further purification as crude extract or by minimal processing. As described by Topp et al. (2016) to be competitive, veterinary vaccines need to have a number of desirable attributes, many of which are met using plant-based production. Many candidate subunit vaccines have been produced in plants (Table 12) and tested in target animals with positive

outcomes (Kolotilin et al., 2004). Based on these considerations, it is indeed surprising to see that 26 years down the road only two recombinant protein products from plants had made it through the regulatory processes to be licensed: monoclonal antibody against HBs-Ag and poultry vaccine against NDV. The idea of plant-made vaccines as edible vaccines has received much publicity and enthusiastic development since the first proof of concept recombinant plant-derived pharmaceutical proteins was reported. However, the progress made was not without hurdles. Although the reports of successful expression of target antigens of interest were numerous, many of these failed to achieve expression levels suitable for commercialization. Besides, the use of food plants for production of vaccine antigens has sprouted fears of contamination of the human food chain. Worries about regulatory issues have also deterred the development of plant-made vaccines.

Characteristic of efficacy	Comments	The plants advantages	References
Subunits antigens	Eliminate safety risks from attenuated live vaccines	Reduced risk of zoonotic pathogens in vaccine	Soria-Guerra et al., 2010
Particulate antigens	Compared to soluble antigens more efficient uptake by antigen presenting cells. Better stability following administration	Ability of plants to produce VLPs and multimeric protein aggregates /protein bodies	Rosales –Mendoza., 2014
Persistence in the gut	Protein needs to tolerate low gastric pH	Plant matrix can confer protection	Pelosi et al., 2012
Ability to cross epithelial barrier	Need to get from GI tract into tissue for antigen presentation	Subunit vaccines can be engineered with cell penetrating moiety or ligands binding to enterocyte receptors	Devriendt et al., 2013
Monoclonal Antibodies	Polyclonally antibodies have batch to batch variability	Antibodies can be engineered –*e.g.* nanobody, glycosiliation , grafting to animal specific Fc	Virdi et al., 2013
Adjuvancy	Increases responses	Subunit vaccines engineered with adjuvant moiety	Rosales Mendoza., 2014
Mode of administration	Oral or nasal easier than parenteral and more effective for mucosal immunity	Plants expressing vaccine antigen offer potential for oral vaccine ; dose calibration is challenging with oral delivery in feed	Mason and Herbst-Kralovetz, 2012
Shelf life	Stable under challenging conditions of temperature , moisture	Longer shelf life	Kwno et al., 2013b
Cost-benefit	Return on investment out weight cost	Low unit cost of plant-based unpurified , orally administered product	Xiao et al., 2015

Table 12: Desirable characteristics of veterinary vaccines and immunotherapy agents (Kolotilin et al., 2004)

However, the regulatory pathway for plant molecular farming of vaccine antigens for veterinary use is far shorter when compared to products intended for human use.

1.12.1-Plant–based vaccines against parasitic diseases

The health and well-being of food-bearing animals is major preoccupation for any livestock producer.

In developing, an edible vaccine is fundamental choosing suitable antigenic proteins expressing in plants. Animal parasitic diseases are an effective problem in livestock and appropriate strategies on order to avoid outbreaks of serious illness are needed. Several parasitic diseases can be kept under control using plant- based vaccines. The various reports in this regard can be summarized as follows.

Fasciola hepatica

Fasciolosis caused by *Fasciola gigantica* and *Fasciola hepatica* is a relentless constraint on the growth and productivity of cattle, buffaloes and sheep (Mehra et al., 1999; Yamasaki et al., 2002). Among the various subunit-vaccine candidates' cysteine proteases (CP) remains the most promising. CP released by *Fasciola spp.* plays a key role in feeding and in the migration through host tissues and immune evasion (Tort et al., 1999) could induce high levels (>70%) of protection in cattle. Recently, Legocki et al. (2000) engineered a 981 nucleotide cDNA fragment encoding the catalytic domain of the cysteine protease of F. *hepatica* into plant transform. After estimating antigen concentration, transgenic *alfa-alfa* and lettuce were fed model mice in order to assess their ability to perform as an edible vaccine as well as to find out safety index. They have immunized mice twice per month with 2 μg of cysteine protease. After the second immunization, there was a significant increase in antigen specific IgG and IgA antibodies in serum. The results suggest that the orally administered antigen in lettuce and *alfa-alfa* was able of inducing an effective immune response in mice.

Taenia solium

T. solium cysticercosis is a major parasitic disease of global proportion that poses a very serious threat to human. Vaccination of pigs, the only intermediate host, could impede the parasite transmission, thus reducing human infection and increase swine health status (Sciutto et al., 2000). Various whole or subunit vaccine candidates with promising protective effects against porcine

cysticercosis have been reported, but their effectiveness has been assessed experimentally rather than under complex field conditions (Wang et al., 2002; Gonzales et al., 2005). A vaccine based on 3 synthetic peptides, KETc1, KETc12 and GK1 consisting of 12, 8 and 18 amino acids, respectively (designated S3Pvac) was shown to be effective in mice against Taenia crassiceps (Toledo et al., 1999; Toledo et al., 2001) and in pigs against *T. solium* (Huerta et al., 2001). These peptides are present in all stages of development of *T. solium*. S3Pvac is the only field trial-tested vaccine candidate against cysticercosis, which was originally, identified in *T. crassiceps* by screening the cDNA library of *T. crassiceps* with sera from *T. solium* infected pigs. S3Pvac is capable of inducing significant protection against cysticercosis in vaccinated pigs, both experimentally and in the field where transmission conditions are most pressing (Sciutto et al., 2007). Insights in cysticercosis prevention and in the development of new antigen delivery systems is useful for the design of more effective and affordable oral subunit vaccines to be feasible applicable in the endemic countries stringent economies.

<u>*Ascaris suum*</u>

A. suum has been identified as a ubiquitous roundworm of swine. Recent studies have emphasized its importance as a zoonotic parasite (Nakamura-Uchiyama et al., 2006). Protection of animals from *A. suum* infection by immunization with third stage larvae (L3) or their cuticle component led to the discovery of a protective 16-kDa antigen (As16) of *A. suum* L3. This antigen was later found to be expressed in the intestine, hypodermis, and cuticles of both larvae and adults (Tsuji et al., 2003). Following these findings, Matsumoto et al. (2015) expressed As16 as a chimeric fusion protein with B-subunit of cholera toxin (CTB) in rice endosperm under the control of endosperm-specific glutelin-GluB-1 promoter. This led to the accumulation of the chimeric fusion protein in seeds up to 50 µg per gram. However, when the transgenic rice seeds were fed to mice they could get only a weak immunogenic response. Therefore, future studies should be directed towards the improvement of *in-vivo* immunogenic potential of this transgenic vaccine.

Eimeria tenella

Coccidiosis caused by intracellular protozoan parasites belonging to the genus *Eimeria* is recognized as an important disease having the greatest economic impact on poultry production. The first live vaccine released to challenge this disease was in 1956. Although vaccination strategies with a-virulent or attenuated *Eimeria* strains have been routinely used for 50 years, the large-scale production is relatively laborious and expensive. Limited progress has been achieved towards the development of subunit or recombinant vaccines, the major hurdle being the identification of protective antigens and delivery of recombinant vaccines (Shirley et al., 2005; Dalloul et al., 2005). Sathish et al. (2011) expressed one of the microneme proteins, EtMIC2 of *E. tenella* in tobacco leaves using *Agrobacterium*. Feeding of this transgenic plant in poultry resulted in high antibody production along with the reduction in oocytes output. Moreover, the weight gain was considerably higher in these birds compared to control.

Here is increasing evidence that orally applied recombinant antibodies have the capacity to reduce the infectious load in animals following oral administration. Issues such as stability in the gut, tissue penetration, clearance, general immunogenic effects as well as technical issues and commercial applicability have to be addressed in future. Current results indicate that there is no generally applicable "ideal" plant system for the expression of antibodies and antibody fragments, but such systems must be carefully chosen and tailored to the specific type, and even to the specific sequence, of the antibody under study.

1.12.2-Plant-based vaccines (PBV) against bacterial or viral diseases

Oral vaccination has been traditionally viewed as the panacea of plant-produced vaccines in terms of their use as an approach to disease control. In livestock the disease, outbreaks represent significant economic loss not only due to mortality

of the animals, but also for the reduction of the growing performance, and of the feed efficiency, the increase of therapeutic costs and labor. Moreover, in case of zoonotic agents, the potential risk to consumers from contaminated food is an important public health concern. Nevertheless, an estimated 9.4 million cases of

illnesses due to the consumption of food contaminated with known pathogens occurs annually in the United States (Scallan et al., 2011).

1.12.3-PBV against zoonotic diseases

Several plant-employed as edible vaccines were developed and studied against important zoonotic agents. Below some examples are reported.

<u>Viral diseases</u>:

- *<u>Rabies virus</u>*

Rabies is a major public health concern in developing countries as it caused 55000 deaths annually according to WHO report and millions of death in animals (Loza-Rubio et al., 2012). Rabies virus that belongs to the family *Rhabdoviridae* is the major cause of spread infection. Currently, available vaccines are almost useful, however the maintained cold chain at 4 C make this vaccine expensive and present serious limitations in developing countries. Plant-based vaccines give potential solutions to these critical issues (Loza-Rubio et al., 2012). Nucleoprotein of rabies virus transiently expressed, had high-level expression and was antigenic in mice conferring protection against rabies viral challenge (Arango et al., 2008).

- <u>The swine flu virus</u>

One of the most problem in the livestock is swine flu disease in pigs. The virus responsible of infection belongs to the family *Flaviviridae*. Influenza is also a burden for the public health in humans. In fact, has been estimated that millions of people each year need vaccination against flu-virus. Influenza virus infects farm animals and transmits to humans either by direct contact or through contaminated food. Many cases were reported where pig farmers showed symptoms similar to swine influenza after interaction with infected pigs. Oral and plant-based vaccines offer alternative solutions (Jung et al., 2014). E2 structural protein expressed in tobacco chloroplasts conferred protective immune response in mice upon oral delivery (Shao et al., 2008). A further research evidenced that transgenic rice calli expressing E2 structural protein have protective effect in immune system, in orally immunized mice; also pigs develop a specific and systemic immune response against E2-antigenic protein.

- Avian influenza

Avian influenza is a highly infectious and contagious disease that can cause 100% mortality in livestock. As this disease is usually spread in large farms, massive vaccinations are needed to control disease in birds and animals. Plant-based vaccines offer the best solution to control this disease in large animal farms (Firsov et al., 2015). Antigenic protein HA was expressed in endoplasmic reticulum from avian influenza, HPA1 resulted in high level expression; immunogenicity of transgenic *Arabidopsis* was confirmed from orally immunized mice with high level HA specific systematic IgG and mucosal IgA, strong Th-1 responses together with IgG2b production and 72% of immunized mice were protected after viral challenge (Lee et al., 2015).

Bacterial diseases.

Several serious illnesses are also zoonotic infections caused by bacteria. When pathogens gain access to the body serious illness can occur. Routinely the first aim of bacteria is multiplying and crowd out healthy bacteria, after infection bacteria are able to grow in tissues that are normally sterile. Harmful bacteria are particularly able to produce dangerous toxins that damage cells and tissues. Common pathogenic bacteria and the types of zoonotic disease are listed below:

- Anthrax.

An important zoonotic agent related also to the terrorism risks is *Bacillus anthracis*. It is worth to mention that Anthrax outbreaks have been continuously recorded in Asia, Africa and South America. Anthrax is an animal disease, but humans get often the infection during hunting, eating contaminated food or direct contact with animals. Nowadays vaccines against human and animal anthrax are injectable and derived from protective antigen from obtained culture filtrate of *Bacillus anthracis*. There are several limitations to this vaccine, including requirement of several boosters (up to eight) and withdrawal of certain batches due to toxin contamination in the culture filtrate (Koya et al., 2005). Transplastomic tobacco expressing the anthrax protective antigen (PA) was observed to be protective against infection in immunized mice. The animals challenged producing high-titer IgG antibodies against anthrax (1:320000) and get 100% protection after challenge with lethal dose of *Bacillus anthracis*. Protective immune response was confirmed by oral feeding with transgenic

plants and challenge with lethal dose of *Bacillus anthracis*. Orally immunized mice produced IgA, IgG1, IgG2a titers and showed 60%–80% protection after challenge (Gorantala et al., 2014).

- Tuberculosis

Tubercolosis (TB) is a zoonotic disease that causes serious illness. TB has bacterial origin in both animals and humans. In 2010, TB affected 8.8 million people and resulted in 1.5 million deaths. TB has a high mortality rate and represents a severe unhealthy status in humans and animals. In evolved countries, TB has been mostly eradicated. However; it remains a problem in most of the poor countries. *Mycobacterium tuberculosis* is the etiological agent of TB in humans, on the other hand bovine get TB infection with *M. bovis*. Both species are closely related. *M. bovis* is risky in humans as 10% TB infection in humans is zoonotic due to *M. bovis* (Müller, 2013). The only available vaccine for tuberculosis is BCG, but several limitations remain. The major challenge is drug-resistant TB. Plant-based vaccines could offer potential solutions. Transgenic modified carrot with *Mycobacterium tuberculosis* genes *cfp10, esat6* and *dIFN* produced very low levels of antigen (0.035% TSP), and orally immunized mice with transgenic carrot produced both cell-mediated and humoral immune responses (Permyakova et al., 2015). Chloroplast transformation of CTB fused ESAT6 and Mtb72F in tobacco and lettuce leaves produced much higher level of expression (up to 7.5% TSP) and lyophilized plant cells could be stored at ambient temperature for several months, thereby eliminating the cold chain and this could facilitate development of an affordable vaccine (Lakshmi et al., 2013).

- Listeriosis

Listeria monocytogenes is responsible to induce Listeriosis, an infectious zoonotic disease in humans and animals. Listeriosis is a serious illness caused by a bacterium. This disease is transmitted to humans through contaminated or raw food. The infection is particularly serious in newborn, pregnant women and immunosuppressed people. The infection can induce complications such as encephalitis. Listeriosis is an overlooked zoonotic pathogen and plant-based is a promising way to overcome this disease, and to find the best solution. Transgenic potato based vaccine against Listeriosis showed a very promising result in

immunized mice. A significant reduction of numbers of bacteria was observed in spleen and liver. The immunized mice get better healthy status, even if an infection of *Listeria* occurs (Ohya et al., 2005).

1.12.4-PBV against specie-specific pathogens

The goal for researchers is a great reduction of animal morbidity and mortality and the use and employment of good production practices. The production system "drives" the use of best manufacturing practices. Nowadays, a problem in animal health toolbox are antibiotics. It can be reasonable to assume that the availability of antibiotics will become increasingly constrained as legitimate public alarm. Public opinion and the governements of several countries fear the onset of catastrophic antibiotic resistance in human and veterinary medicine. Based on some issues it becomes pivotal developing new vaccines and immunotherapeutic agents. The use of these new therapies could evolve in a new strategy to challenge diseases. Without using antibiotics against disease, in addiction offer veterinary practitioners much needed tools (Potter et al., 2008).

Edible vaccines offer several advantages over conventional methods of vaccine delivery. Edible vaccines induce primary the lining of the digestive tract, thus enabling them to stimulate both mucosal and systemic immunity, the former of which is a response that is notoriously difficult to achieve through injections, which bypass mucosal barriers. In part, this accounts for the reason as to why the efficacy of injectable vaccines against intestinal pathogens has generally been quite poor. Edible vaccines also have positive implications for livestock welfare, as they could be administered directly in the diet, eliminating the need to confine the animal, or the need to breach the skin through injection, a practice that can promote secondary infections. Oral administration of vaccines eliminates the risks that injection-based methods pose on meat quality as a result of intramuscular administration (Kolotilin et al., 2004).

Below some examples of innovative plant based vaccines that are developed and studied against common animal pathogens are reporded.

- Foot and mouth disease

Rao et al. (2015) produced a bivalent vaccine against foot and mouth diseases expressing VP1 structural protein from two serotypes A and O. The pigs

administered with transgenic plants developed specific humoral immune response. Sera from immunized pigs when challenged with FMDV neutralized the virus with no cytopathic effect on BHK-21 cells (Rao et al., 2015). A further proof, VP1 protein was expressed in tobacco chloroplasts (Lentz et al., 2010). In addiction in several published papers we can found new strategies to express BVDV in tobacco (Nelson et al., 2012), *alfa-alfa* (Aguirreburualde et al., 2013) and *Panax ginseng* (Gao et al., 2015). The immune studies in different animal models showed promising results.

- Canine parvovirus

Causes infection in dogs and particularly in puppies. Currently live and killed virus-based vaccines offer the only solution to treat dogs with CPV infection. Live vaccines are always associated with pathogen of low virulence that can be hazardous for dogs with weak immune systems. Many efforts in the field of plant-based vaccines have been made to find alternate solution to treat or prevent CPV in dogs. Immunization of mice with CTB 2L21 and GFP 2L21 peptide from (CPV) VP2 protein (Molina et al., 2004) and 2L21-TD (Ortigosa et al., 2010) expressed in chloroplasts generated high titers of IgA, IgG1 antibodies against viral VP2 protein.

- Transmissible gastroenteritis coronavirus (TGEV)

The use of maize seeds as an edible delivery vehicle has been extensively studied. The envelope spike protein was used as an antigen to raise neutralizing antibodies and the efficacy of this plant-made vaccine has been presented in experiments with piglets (Lamphear et al., 2004). In addition, it was found that the antigen was stable during storage in various conditions and authors were able to concentrate the antigen using milling techniques.

- Bovine Rinderpest virus

Transgenic peanut plants expressing hemaglutinin raised immune responses in cattle (Khandelwal et al., 2003) This oral vaccine was able to raise virus-specific antibodies which also neutralized the virus *in-vitro*. Immunogenicity of a Tobacco mosaic virus (TMV)-based vaccine against bovine herpes virus (BHV) was studied in cattle (Perez et al., 2003). Immunogenic glycoprotein D was produced as a by-product in TMV-inoculated tobacco plants, and the crude plant extract emulsified in oil and subsequently injected into cattle was able to raise

specific humoral and cellular immune responses. Most importantly, cattle were protected against BHV to similar levels as those vaccinated with the commercial vaccine. As mentioned before, plant-made oral vaccines possess exceptional skills against serious illness in human and animals the most desirables attributes are long-term storage and stability at room temperature and are needles and syringes free. New techniques and concepts are needed to contribute to this growing field of Molecular Farming. In table 13, an overview of vaccines antigens against zoonotic and non-zoonotic diseases is reported.

Disease	Expressed antigen	Expression system	Host	Expression level	Immune response	Ref.
Rabies Virus	-PA -G-protein of RV	Transplantomic Transgenic	Lettuce Carrot	7% TSP 0.4- 1.2% TBS	66% of immunized mice showed protection against virus challenge	Rasoulicet al., 2014
	-G-protein fused with CTB, -G protein	Transient Transgenic	Tobacco Maize	0.4% TBS 25.1g/g of fresh seeds	50-83% protection was observed in immunized sheep	Loza Rubio et al., 2012
Swine flu	-E-2 glycoprotein	Transgenic	Tobacco	1-2% TSP	Orally immunized (mice) : no specific response Sc: CSFV-specific serum IgG	Shao et al., 2008
Bird flu Avian Influenza Virus (AIV)	-NA gene of H1N1	Transgenic	Lettuce	0.018%–0.045% TSP	Orally immunized (mice): anti-NA antibodies at third booster; no virus challenge assay was performed	Liu et al., 2014
Newcastle Disease Virus (NDV)	-HN gene	Transgenic	Tobacco	0.069% TSP	anti-HN serum IgG, no virus challenge study was performed	Hahn et al., 2007
Antrax (*Bacillus antracis)*	-PA	Transplantomic	Tobacco	4.5%TBS	Immunized mice produce antibodies against rabies. and Protection: 66% immunized mice	Koya et al. 2005
Tuberculosis (*Mycobacterium tuberculosis*)	Ag85B, MPT83, MPT64, ESAT6	Transgenic	Potato	Not reported	Immunized mice produced high-titer IgG and IgA antibodies against antigens, and stimulated CD4+ and CD8+	Zhang et al., 2012
(Listeriosis) *Listeria monocytogenes*	IFN-α	Transgenic	Potato	Not reported	Orally immunized mice with transgenic IFN-α showed its ability to reduce bacterial burden	Ohy*a* et al., 2007
Pasteurellosis	GS60	Transgenic/Transient	Alfalfa/ Tobacco	0.02% TSP	Immunized rabbit with transgenic alfa-alfa produced antibodies against GS60	Lee et al., 2008
Brucellosis	U-Omp19	Transient	Tobacco	Not reported	Immunized mice produced specific response	Pasquevich et al., 2011

Table 13: Vaccines antigens against zoonotic and non-zoonotic diseases. PA: protective antigen. TSP: total soluble proteins.

1.13-Escherichia coli infections in pig livestock

The pathogenic strains of *Escherichia coli* recovered from the intestinal tract of animals fall into categories called enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC) and necrotoxigenic. The pathogenicity of these strains is determined by the presence of certain genes that encode adhesins and toxins, are generally organized in large blocks in chromosomes, large plasmids or phages, and are often transmitted horizontally between strains.

ETEC strains are equipped with adhesion factors and enterotoxins: they are heat-labile and heat-stable. The EPEC strains also produce intestinal lesions, but act through mechanisms distinct from those of ETEC and EHEC. EHEC strains are so called because the prototype of this group, serotype O157: H7, causes in humans, hemorrhagic colitis. As it produces cytotoxic toxins (Verotoxin, VT), the term "enterohemorrhagic" (EHEC) and "verocitotoxic" (VTEC) were often overlapping.

Among VTEC, serotype O157:H7 has been closely associated with the sporadic and clinical outbreaks of hemorrhagic colitis, hemorrhagic uremic syndrome, and thrombotic thrombocytopenic purpura in human beings (Croxen et al., 2010). Although ruminants, primarily cattle, have been suggested as typical reservoirs of STEC, many food products of other origins, including pig products, have been confirmed as vehicles for STEC transmission. In swine, STEC plays an important role in the pathogenesis of enterotoxiaemiae. In particular, a Shiga toxin subtype, named stx2e, it is considered as a main factor responsible in the damage of swine endothelial cells. On the contrary, stx2e-producing *Escherichia coli* has rarely been isolated in humans, and usually only from asymptomatic carriers or from patients with mild symptoms, such as uncomplicated diarrhoea. In fact, the presence of gene stx2e, encoding for stx2e, has rarely been reported in STEC strains that cause HUS. Moreover, stx2e-producing STEC isolated from humans and pigs were found to differ in serogroups, their virulence profile and interaction with intestinal epithelial cells. Because of the limited epidemiologic data of STEC in swine and the increasing role of non-O157 STEC in human illnesses, the relationship between swine STEC and human disease needs to be further investigated (Ercoli et al., 2015).

From the clinical point of view, the swine colibacillosis can be divided into intestinal and extra- intestinal infections (Table 14). The first manifest themselves in the form of diarrhea (neonatal and pre-or post-weaning), of hemorrhagic gastroenteritis or in enterotoxaemia form. The latter include instead septicemia and polyserositis or, rarely, mastitis, cystitis and pyelonephritis (Zavanella et al., 2009).

Pathology	Strains
Neonatal diarrhea	O8, O9, O101 O20, O64, O147, O149, O157
Pre-weaning diarrhea	O8, O115, 0147
Post-weaning diarrhea	O8, O9, O115,0138, O139, O141, O147, O157
Hemorrhagic gastroenteritis	O8, O149, O157,
Oedema diesasse	O138, O139, O141

Table 14: Strains of *E. coli* associated to the diseases in pigs (Zavanella et al., 2009)

In particular, regarding the enterotoxaemia, attributable to verocytotoxic *E. coli* (VTEC) strains, it occurs in piglets (about one or two weeks after weaning, while they are six-fourteen weeks old) but it is also reported in adult pigs.

PWD is caused by $F4^+$ and/or $F18^+$ enterotoxin-producing *E. coli* (ETEC), while OD is the result of an infection with verotoxin-producing $F18^+$ *E. coli* (VTEC). Up to now, no vaccine protecting piglets against these infections is available and treatment relies upon the use of antimicrobials.

1.13.1-O138 verocytotoxic E. coli in weaned piglets

Among the different phases of production, the weaning transition is a particularly complex period, during which the piglets are confronted by multiple stressors. Weaning involves complex social changes, including abrupt separation from the sow, a new housing system, separation from littermates and mixing with other litters in a new environment. Diet composition also changes at weaning: the liquid highly digestible milk from the sow is replaced by a less digestible and more complex dry feed. Early weaning (21 days or before) in intensive production systems is more likely to exacerbate the level of general stress, contributing to increased mortality, decreased growth performances and abnormal behavior of piglets (Kil et al., 2010).

The immediate effect of weaning is a dramatic reduction in feed intake and a consequent ‘growth check’ (Pluske et al., 1997), which continues to represent a major source of production losses in commercial piggeries. Weaning is a critical

stage of pigs production and is associated with changes in the structure and function of the gastrointestinal tract. Intestinal alterations often seen in newly weaned piglets include reduction in villous height and increased crypt depth, reduced activities of intestinal digestive enzymes, disturbed intestinal absorption, secretion and permeability (Boudry et al., 2004; Lalles et al., 2004). Different stressors, related to the management may affect the immune fuction and the intestinal microflora of pigs, increasing the risk of enteric disorders.

After the ban of in-feed antimicrobial growth promoters (AGP) in 2006, traditionally used in newly weaned pigs to control enteric pathogens, new strategies are required. *E. coli* is one of the main causes of important economic losses in the livestock industry, especially for the pigs: a group of diseases related to many factors, such as environmental, managerial, hygienic, and nutrition.

Verocytotoxigenic *E. coli* (VTEC) was the first identified as a distinct group of *E. coli* named as verocytotoxigenic *E. coli* (VTEC), which had the ability to produce toxins with profound and irreversible effect on Vero cells (Reuter et al., 2016). VTEC is also termed as Shiga-like toxin producing *E. coli* or Shiga toxin producing *E. coli* or STEC. Acronym STEC is derived from the fact that the toxins are Shiga like that is similar to those produced by *Shigella dysenteriae* Type 1 (O'Brien et al., 1987).

Clinical signs of enterotoxaemia occur with oedema disease, intense diarrhea, and dehydration: the perineum looks yellowish and watery feces smeared; the stomach looks distended and contains undigested milk, the intestine is dilated and with a thickened wall, filled with clear liquid, and the mucosa is reddened and full with watery material containing.

In farming industry verocytotoxic *Escherichia coli* (VTEC) is an important pathogen, causing serious mortality and severe production losses (Rossi et al., 2013). Two crucial virulence factors are present: (1) adherence factors (often fimbriae) in order to mediate the attachment to specific receptors, usually glycans, followed by colonization of the intestinal tract and (2) the production of one or multiple toxins that induce disease symptoms. In piglets, VTEC strains expressing F18 fimbriae (Figure 4) are associated with post-weaning diarrhea

and enterotoxiaemia. The major protein of the F18 fimbria, FedA, is not sufficient for recognizing the F18 receptor (Imberechts et al., 1992). Two additional genes from the fed gene cluster, fedE and fedF, have been described as essential for fimbrial adhesion and fimbrial length (Imberechts et al., 1996). However, so far it has not been possible to assess F18 adhesion function with regard to either of the two gene products.

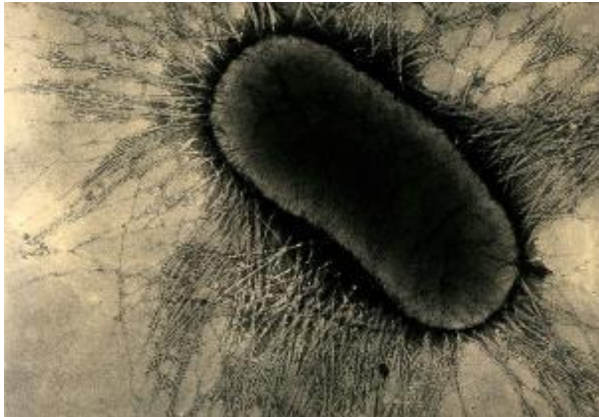


Figure 4: *E. coli* expressing adhesive F18 fimbriae (electronic microscopy), (Imberechts et al., 1996).

After the initial adherence step via the F18 fimbriae VTEC strains produce and secrete the heat-labile (LT) and/or heat-stable enterotoxins (ST), thereby stimulating the secretion of electrolytes and water and resulting in dehydration of the enterocytes and watery diarrhea (Sears et al., 1996; Nagy et al., 2005) (Figure 5).

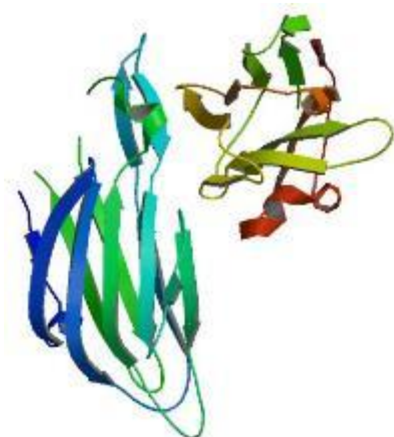


Figure 5: Shiga-like toxin. The toxin has two subunits designated A-B. The B subunit is a pentamer that binds to specific glycolipids on the host cells, a globotriaosylceramide (Gb3). Following this, the A subunit is actuve enzimaticaaly (Rao et al., 2011).

O138 *E. coli* is a F18 positive VTEC strain producing the Shiga toxin Stx2e. Damage to the vascular endothelium eventually results in edema, hemorrhage and microthrombosis, and will be fatal in 90% of all affected animals.

F18 fimbriae are assembled by dedicated machinery, the chaperone/usher (CU) pathway, which is distributed among genera of the phyla Proteobacteria, Cyanobacteria, and *Deinococcus-Thermus*. Essential to the CU pathway are a periplasmic chaperone protein and an outer membrane pilus assembly platform, termed usher (Busch et al., 2012). Fimbriae subunits or pilins are stabilized by the chaperone and complemented in the final quaternary structure by an N-terminal donor strand of the following subunit. The usher both acts as a building platform and anchors the fimbriae to the cell surface (Phan et al., 2011). Crystal structure of the FimD usher binds to its cognate FimC-FimH substrate. The two-domain tip subunit often harbors the adhesive properties and thus determines the host tropism of the bacteria (De Greve et al., 2007). It features a typical two-domain organization comprising an N-terminal receptor-binding domain linked to a C-terminal pilin domain that forms the connection to the fimbrial shaft. F18 fimbriae are comprised of the major subunit FedA (Imberechts et al., 1992), the minor subunit FedE and the tip-adhesin FedF (Smeds et al., 2001), which binds to glycosphingolipids having A/H blood group type 1 core antigens (H: Fucα2Galβ3GlcNAcβ3Galβ4Glc;GalNAcα3) that are present on enterocytes of the small intestine (Coddens et al., 2002). Two subtypes of the F18 fimbria are distinguishable by serologic methods: F18ab (formerly, F107 fimbria) and F18ac (formerly, 2134P, 8813, or Av24 fimbria) (Rippinger et al., 1995).

Both F18 fimbrial subtypes appear correlated with different pathovars and O antigens. O antigens are constituted by certain sugar residues of lipopolysaccharides present in the outer membrane of Gram-negative bacteria. Although O antigens appear not directly involved in bacterial virulence, serotyping is often used in routine diagnostics for presumptive identification of virulent strains among porcine *E. coli* isolates (Gyles et al., 2010). While F18ab fimbriae have been found predominantly in STEC exposing the O138 or O139 antigen on the bacterial surface, F18ac fimbriae were reported dominant in ETEC strains of serogroups O141, O147, and O157 (Hyde et al., 1995; Nagy et al., 2005).

Pathogenesis

After birth, suckling piglets are protected from F18$^+$ VTEC by specific antibodies in the sow colostrum. Vaccination of the sow can enhance the protection and so far, several maternal vaccines are available on the market (Cox et al., 2002). At weaning this lactogenic immunity is lost and together with the stress associated with the weaning period the piglet will become highly vulnerable to infection.

VTEC can survive the high acidity of the stomach and with such small numbers capable of causing disease, infection can occur without any growth of the bacteria in food. The incubation period for VTEC infection ranges from 1–8 days but is typically between 2 and 4 days (Maule, 2000). The relatively long incubation period of VTEC has significant implications for investigation. It makes recall bias (inability to remember accurately what foods were eaten or where the patient was during the possible exposure period) more likely (report of the Health Protection Surveillance Centre (HPSC) subcommittee on verotoxigenic *Escherichia coli*, 2005).

Ingestion of VTEC is followed by colonization of the intestine in pigs in which intestinal epithelial cells carry receptors for the F18 pili. Expression of the receptors is related with age; in fact, younger pigs are less susceptible to colonization than older pigs. Some pigs with a specific mutation in a gene that codified the receptors are resistant to infection. Resistance/susceptibility is established by a single locus with a dominant susceptibility allele and a recessive resistant allele; it is allowing selection of resistant pigs, which can be analyzed by a simple PCR test that identifies the presence or absence of the specific mutation. Some ideas about selection for resistance to F18$^+$ *E. coli* has been expressed, because a very high association between the presence of the marker for resistance to F18$^+$ *E. coli* and the presence of the marker for stress susceptibility was shown in Swiss Landrace pigs.

VT2e proteins in the intestine of colonized pigs are responsible for the major clinical signs and pathology symptoms. O138 *E. coli* may colonize the mesenteric lymph nodes and produce VT2e there. In addiction, this site the toxin is absorbed into the bloodstream. The VT2e toxin binds readily to pig RBCs,

which may deliver the toxin to various sites in the body. Sites highly susceptible to the toxin include the submucosa of the stomach, the colonic mesentery, the subcutaneous tissues of the forehead and eyelids, the larynx, and the brain. Damage to vascular endothelium results in edema, hemorrhage, intravascular coagulation, and microthrombosis.

High-protein diets increase the susceptibility of pigs to the disease. Factors associated with weaning, including the stresses of mixing pigs, changes in diet, and the loss of milk antibodies from the intestine, appear to be important elements in enhancing the susceptibility of weaned pigs to the disease.

Currently, no vaccine is available to protect pigs against VTEC. Expressed antigens in plants against post- weaning disease offer potential solutions. Orally immunized mice and pigs with tobacco seeds expressing VT2e-B and FedA conferred protective immune response against O138 *E. coli*, and immunized pigs showed protection against O138 *E. coli* infection (Rossi et al., 2014).

In this study, two proteins actively involved in the pathogenesis of the disease were selected as potential immunogens against O138 *E. coli* infectio*n*: the F18 fimbria, responsible for the adherence of the bacteria on small intestinal enterocytes, and the B subunits of VT2e, responsible for binding the toxin to specific receptors on the cells surface.

2-AIM OF THE STUDY

This work focused on *Nicotiana tabacum*-based vaccines engineered for the expression of antigens against verocytotoxic *Escherichia coli* (VTEC strain). In a previous study, tobacco plants of *Nicotiana tabacum* specie were transformed via agro-infection for the seed-specific expression of the antigenic proteins.

The general aim was to define the strategy to induce effective local immune response in weaned piglets by oral administration of engineered tobacco seeds.

In particular, the following issues have been investigated:

✓ Evaluation of germination, growth and development of vaccinal plants in Fitotron and greenhouse: seeds of two antigenic lines ($F18^+$ and VT2e-B^+) and wild-type of tobacco were seeded and monitored.
Optimization of harvesting seeds practices and quantification of seeds from transgenic tobacco plants.

✓ Chemical, molecular and morphological evaluation of engenireed plants. In particular, the stable integration of transgenes, their expression and their influence on principal morphological characteristics were investigated.

✓ Definition of experimental conditions in order to perform the oral immunization of piglets. In particular, the palatability, dosage, texture, milling procedures were investigated.

✓ Evaluation of the protective effect of immunogenic seeds throught *in-vivo* trial (ethical authorization: 102/2015PR – Health Ministry) carried out at the experimental animal research and application Centre of the University of Milan in Lodi.

In order to examine in depth, the aspects related to local immunity, an independent project to characterize the effects of different mucosal bacterial pathogens (two strains of *H. influenzae, M. catarrhalis and S. aureus)* on the isotype of antibody production from human B cells has been developed. The potential "decoy" stimulation by surface bacterial proteins that is proposed to stimulate B cells non-specifically by cross-linking surface antibody by the constant Fc region rather than binding these bacteria by the antigen-specific Fab variable region. This part of the study has been conducted in collaboration with the Department of infectious diseases, University of Denver, Colorado.

3-MATERIALS AND METHODS

ABBREVIATIONS:

dNTPs	deoxynucletide triphosphate
IPTG	Isopropyl β- D -1-thiogalactopyranoside
SDS	Sodium dodecyl sulfate
RT	room temperature
o/n	over night
bp	base pairs
rpm	revolutions *per minute*
OD600	Optical Density, of a sample measured at a wavelength of 600
nm	nanometer
PBS-t	Phosphate Buffered Saline adding of the detergent Tween 20
LB	Luria-Broth
KAN	Kanamycin
kDa	KiloDalton
TMB	3,3',5,5'-Tetramethylbenzidine
BSA	Bovine Serum Albumin
NDF	Neutral Detergent Fiber
ABTS	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
BHI	Brain Heart Infusion
IFN-γ	Interferon-γ
PBMC	peripheral blood mononuclear cell
IL-21	Interleukyn-21
TSB	Tryptic Soy Broth
HRP	Horseradish Peroxidase

3.1-Vaccinal biomass production

In the present study, two lines of *Nicotiana tabacum* plants, previously engineered for the seed-specific expression of the major subunit Fed-A of the F18 adhesive fimbriae and VT2e B-subunit genes respectively, were considered as model of edible vaccines against porcine verocytotoxic *E. coli* (Rossi et al., 2013). Briefly, tobacco plants were transformed via *Agrobacteium tumefaciens* with chimeric constructs containing structural parts of genes under control of a seed specific GLOB promoter (Figure 6).

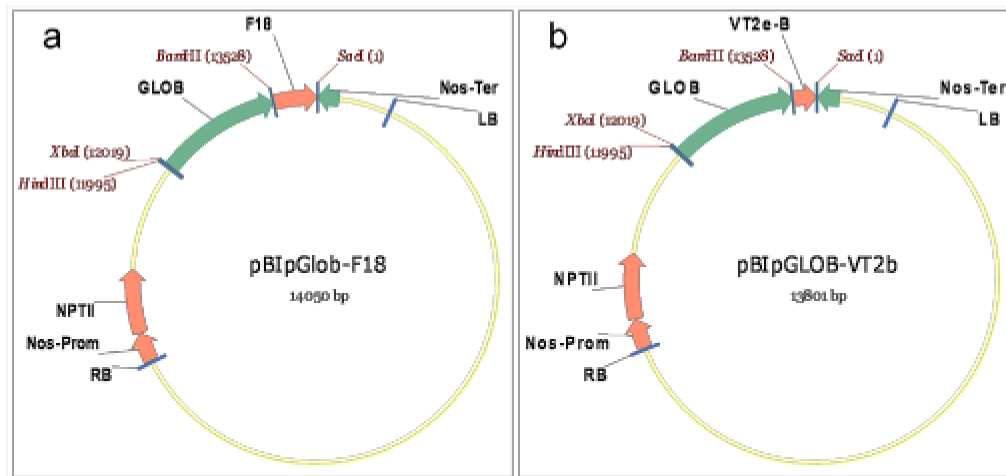


Figure 6: Chimeric constructs used for *Agrobacterium tumefaciens* EHA105 transformations. Transgenes were inserted under control of the GLOB promoter and *NOS* terminator. (A) pBIpGLOB-VT2e-B was 13800 bp. (B) pBIpGLOB-F18 was 14049 bp.

3.1.1-Seeding

Three lines of tobacco seeds *(Nicotiana tabacum L., cv. Xanthi*), two transgenic (F18$^+$ and VT2e-B$^+$) and one wild type used as control, were seeded in three seedbeds with growing soil (sphagnum, peat, lapilli and pumice) previously wetted for 24 hours on 2 cm of water.

In particular, three tobacco seeds were potted in each pots of seedbed, using spatula and microscope (Figure 7). After sowing directly onto soil, the seeds were wetted with normal water three days a week to keep them humid.

A total of 80 pots per each vaccinal line (F18$^+$ and VT2e-B$^+$) and 56 pots for wild type were seeded.

Seeds were maintained in a Fitotron® growth chamber in controlled environmental condition (25°C ± 2°C; 14 hours of light by 400 W high-pressure sodium lamps and 10h dark).

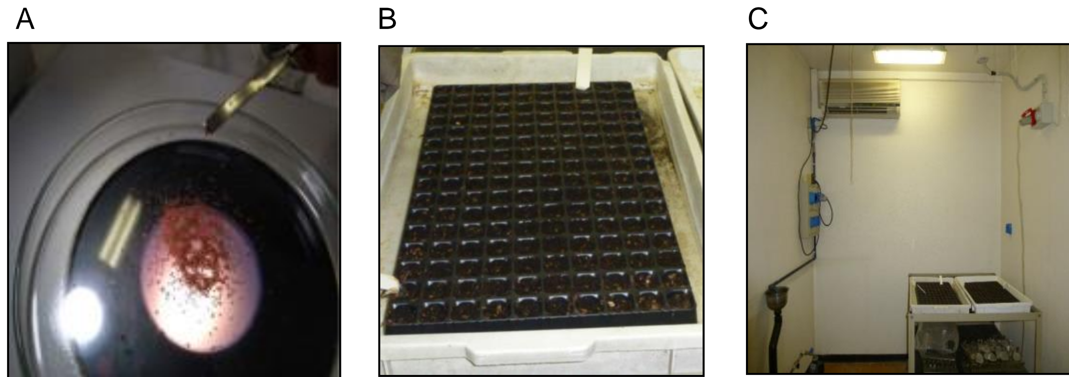


Figure 7: Times of seeding of transgenic seeds and wild type seeds. A: seeds were selected by a spatula and using microscope; B the seedbed with commercial soil; C: Fitotron growth chamber at optimal growth conditions

3.1.2-Germination and morphology evaluation

The germination was determinate by index. The germination index was measured with the following formula to evaluate and compare the germination value and time of control, F18 and VT2e-B plants.

% of germinate plants= number of germinated plants/total number of pots

After the germination the height of the out-soil part of the plants were monitored weakly for 50 days.

3.1.3-Cultivation in the greenhouse

80 plants for each vaccinal line (height 15 cm) were transferred at the Orto Botanico G.E. Ghirardi in Toscolano Maderno (Brescia), a botanical garden of the University of Milan, in collaboration with the Department of Bioscience. The plants were maintained in a greenhouse using biosafety level 1, according to the 98/81/EC Directive.

In the greenhouse, plants were stored and isolated from the environment by the polyethylene monofilament to avoid cross-pollination and to provide protection against aphids, white fly and *Bemicia tabaci*, as shown in Figure 8.

The environmental conditions were maintained: temperature between 25-30°C, 80-85% atmospheric humidity, a low level of nitrogen in the soil, a photoperiod of 14h light and 10h dark by a specific lamp. The plants were treated with water containing 20% of total nitrogen, 20% of Phosphate (P_2O_5) and 20% of Soluble Potash (K_2O) Peters Professional® (by Scotts Company).

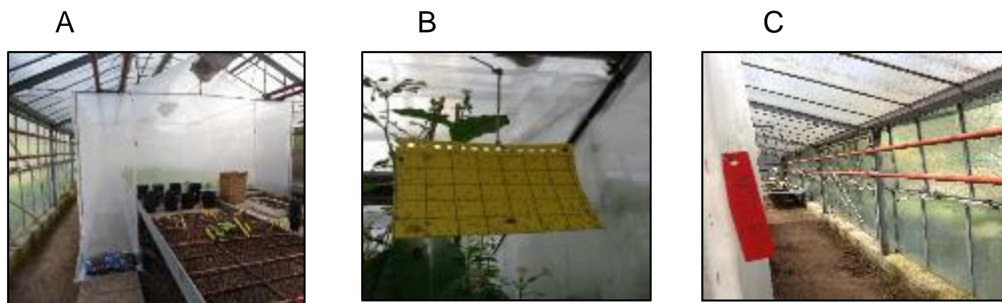


Figure 8: Level 1 of containment in the greenhouse. A: experimental plants harvested under a polyethylene monofilament that provides protection from aphids, white fly and *Benicia tabaci;* B: Sheet for insect killer; C: The plants growth in separate room to avoid cross-pollination.

3.2-Seed collection

After the flowering period, brown ovaries were cut off and collected. Seeds ovaries were stored in a cool, dry place to air dry for 7-10 days. After this period, each ovary was opened in order to collect transgenic seeds. The harvest of seeds was performed in different rooms to avoid contamination between two vaccinal lines: $F18^+$ and $VT2e\text{-}B^+$.

Seeds were sifting and separated from debris and residual part of ovary with Vibroscreen Retch by using sieves with different diameter (Figure 9) for 4 minutes and amplitude 50. The seeds were stored for next experimental trial and to confirm the presence of the transgene in the progeny (R_3).

Figure 9: Vibroscreen used after seed collection in order to separate seeds from capsulaes.

3.3-Chemical analyses of principal components of vaccinal tobacco seeds

The chemical analysis of tobacco seeds was performed to measure the principal components: Crude protein (CP), according to the official method of Analysis of Association of Analytical Communities, procedure 2001.11 (AOAC, 2005); dry matter (dm), according to procedure 930.15 (AOAC, 2005); fat (EE) according to DM 21/12/1998; crude fiber (CF), according to procedure Ba 6a-05 of the official method of the American Oil Chemists Society (AOAS, 1998); ash, according to procedure 942.05 (AOAC, 2005).

3.4-Molecular Analyses of tobacco plants

3.4.1-DNA extraction from seeds and leaves

In order to confirm the presence of F18 and VT2e-B genes in the two vaccinal lines and the absence of the genes in the controls, DNA was extracted from leaves and seeds (20% of growing plants and 20% of obtained vaccinal seeds).

In particular, 40 mg of each sample was grinded into a fine powder freezing it with liquid nitrogen by mortar and pestle. For the isolation genomic DNA from plants, tissues and seeds, the *Wizard*® Genomic DNA Purification Kit by Promega (Promega Corporation 2800 Woods Hollow Road Madison, WI 53711 USA) was used. Extracted DNA was quantified by a 0.8%-1.5% agarose gel and by a spectrophotometer. In the agarose gel, DNA samples were compared with PCR 100 base pairs (bp) Low Marker (SIGMA-Aldrich, St. Louis MO USA).

3.4.2-Detection of F18, VT2e-B and NOS genes

F18 gene was detected with PCR using the two primers reported in table 15. PCRs were performed in 25-µl volumes containing 5 µl of DNA template, 0.2 mM concentrations of deoxynucleoside triphosphates, 2.5 µl of 10X PCR buffer II, 3 mM MgCl2, 1 µM concentrations of each forward and reverse primer, 1.25 U of AmpliTaq Gold (Perkin Elmer). The thermal cycling conditions were as follows: 1 cycle of denaturation at 95°C for 5 min; 35 cycles of melting at 95°C for 1 min, annealing at 56°C for 1 min 20 s, and elongation at 72°C for 1 min 30 s; and a final extension at 72°C ∞.

Gene		Oligonucleotide sequences	PCR product size (pb)
F18 adhesive fimbriae	313 F 314 R	5'ggatccatgaaaagactagtgtttatttcttttg 3'cgaatgcgccaatgaatgttcattctcgag	519

Table 15: Sequences of oligonucleotides for F18 detection.

VT2e-B gene was detected by polymerase chain reaction using two primers reported in table 16. PCRs were performed in 25-µl volumes containing 5 µl of DNA template, 0.2 mM concentrations of deoxynucleoside triphosphates, 2.5 µl of 10X PCR buffer II, 3 mM MgCl2, 1 µM concentrations of each forward and reverse primer, 1.25 U of AmpliTaq Gold (Perkin Elmer). The thermal cycling conditions were as follows: 1 cycle of denaturation at 95°C for 5 min; 35 cycles of melting at 95°C for 1 min, annealing at 50°C for 1 min 20 s, and elongation at 72°C for 1 min 30 s; and a final extension at 72°C ∞.

gene		Oligonucleotide sequences	PCR product size (pb)
VT2e-B subunit	307 F 308 R	5' ggatcc**a**tgaagaagatgtttatagcgg 3' aacgggtccacttcaaatgattctcgag	270

Table 16: Sequences of oligonucleotides for the VT2e-B detection.

Primers HA-*NOS*118-f and HA-*nos*118-r (Lipp et al., 2001), reported in the table 17, were used for the detection of the *NOS* terminator. Amplification of the NOS terminator will result in the production of a DNA fragment of 118 bp.

gene		Oligonucleotide sequences	PCR product size (pb)
NOS terminator	118 F 118 R	5' gcatgacgttatttatgagatggg 3' gacaccgcgcgcgataatttatcc	118

Table 17: The oligonucleotide sequences for NOS detected by PCR.

PCRs were performed in 50-µl volumes containing 15 µl of DNA template, 0.2 mM concentrations of deoxynucleoside triphosphates, 5 µl of 10X PCR buffer

II, 3 mM MgCl2, 1 μM concentrations of each forward and reverse primer, 1.25 U of AmpliTaq Gold (Perkin Elmer). The thermal cycling conditions were as follows: 1 cycle of denaturation at 95°C for 10 min; 50 cycles of melting at 95°C for 25 s, annealing at 62°C for 30 s, and elongation at 72°C for 45 s; and a final extension at 72°C ∞.

PCR-product were evaluated on agarose gel (F18 0.8%, NOS: 1%; VT2e-B: 1.5%).

3.5-Evaluation of the expression of antigenic proteins in seeds

The seed expression of antigenic proteins was evaluated through ELISA assay in collaboration with PLANTECHNO S.R.L.

3.5.1-Positive controls: purified antigens

The F18 protein was purified according to the protocol previously described by Goddeeris et al. (2002). Briefly, O138 *E. coli* strain (F18 positive) was cultured in Tryptone Soya Broth at 37° C and 85rpm for 18 h. Subsequently, the bacteria were collected by centrifugation (3000×*g*; 35min) and washed in PBS, after which the F18 fimbriae were isolated by heat shock (60° C for 20 min). Larger fragments were removed by centrifugation (10,000×*g*; 20 min) and the supernatant was further purified by a subsequent centrifugation (20,000×*g*; 40 min), both at 4°C. The solubilized F18 fimbriae was precipitated with 20% (w/v) ammonium sulfate and the pellet was dissolved and dialyzed overnight against ultra-pure H_2O.

VT2e-B protein was produced by pET-system (Novagen) in *E. coli* BL21 as described in Rossi et al. (2013). In particular, *E. coli* BL21 competent cells (Novagen) were transformed with the expression plasmid pET-28a containing the VT2e-B gene and selected on LB-Kan plates (kanamycin 30 ug/mL). A single recombinant colony was inoculated with 5mL of the LB medium containing kanamycin (37°C, o/n). A total of 1 mL of the pre-inoculum was added to 100 mL of the LB medium in a 1L flask, and incubated with shaking (175 rpm at 37°C) until the OD_{600} reached 0.6. Isopropyl-Beta-D-thiogalactoside (IPTG) was added to the culture to a final concentration of 0.1 mM and the

incubation was continued for another 2 h. The culture was incubated for an additional 3 h and then collected by centrifugation. The soluble protein fraction and inclusion body proteins were eluted separately in columns with a specific resin. The proteins were resolved by 10% polyacrylamide gel electrophoresis. Detection was performed by Coomassie blue staining. The purified VT2e-B protein was concentrated in a 4M urea elution buffer with a centrifugal filter (10kD molecular weight *cut-off*, Centricon). The transgenic protein VT2e-B was expressed in inclusion bodies about 1 mg /100 mL of induced culture, after renaturation the purity of the polypeptides reached >80%.

F18 and VT2e-B proteins were evaluated on an SDS page. The purified proteins were stored at −80 °C.

3.5.2-Specific antibodies against antigens

Anti-F18 antibody was produced by GenScript (GenScript 860 Centennial Ave. Piscataway, NJ 08854 USA). The sequence of F18 antigenic protein (Table 18) was analyzed by Genscript's Peptide Antigen Database. Three peptide antigens were identified across the target protein sequence (Table 19). In some instances, peptide antibodies failed to recognize the folded conformation of the native protein. However, generating antibodies against multiple antigens can significantly increase the success rate of endogenous protein detection.

Sequence of F18 protein
MKRLVFISFVALSMTAGSAMAQQGDVKFFGNVSATTCNLTPQISGTVGDTIQLGTVAPSGTGSEIPF ALKASSNVGGCASLSTKTADITWSGQLTEKGFANQGGVANDSYVALKTVNGKTQGQEVKASNSTV SFDASKATTEGFKFTAQLKGGQTPGDFQGAAAYAVTYK

Table 18: The aminoacid sequence of F18 antigenic protein.

Antigen Designed:
peptide 1--> AQLKGGQTPGDFQG :
peptide 2--> LTEKGFANQGGVAN :
peptide 3--> TGSEIPFALKASSN

Table 19: Aminoacid sequences of thee peptides chosed for the production of specific antibodies.

Using these peptides, the PolyExpress™antibody services can produce high quality polyclonal antibodies with guaranteed ELISA titer >1:64000.

Anti-VT2e-B polyclonal antibodies were kindly purchased and produced by Plantechno s.r.l. The specific antiserum was obtained through the immunization of New Zealand rabbits with VT2e-B protein expressed by pET-system (Novagen) in *E. coli* BL21 (DE3) as described by Rossi et al. (2014).

3.5.3-Set-up ELISA assay

The total proteins were obtained from all mature transformed tobacco seeds by homogenization with liquid N_2 in a mortar and protein extraction with the extraction buffer (50 mM Tris, pH 8.5 mM EDTA, 200 mM NaCl, 0.1% Tween 20). Protein content was estimated by a Bradford assay (BioRad, Hercules, USA) using bovine serum albumin as the standard. An indirect ELISA was developed to quantify the best concentration for the coating and titration of immunogenic proteins VT2e-B and F18.

3.5.4-Quantification of F18 in vaccinal seeds

The coating was performing at room temperature for 2 h in carbonate buffer (50mM, Ph 9.6) We have tested serial dilutions starting from an initial concentration of 2 µg/mL to 60 ng/mL, from 1 to 6 (Table 20), the line 7 represented the negative control. Different dilutions of the primary antibody were tested, starting from a 1:1000 up to a 1:64000 (line A-G) negative control (line H) without antibody. The conditions of the secondary antibody (anti-rabbit peroxidase conjugate) are fixed, dilution 1: 15000, as suggested by the manufacturer (Sigma).

Col	1 2*	2 1*	3 0.5*	4 0.25*	5 0.12*	6 60**	7
A	3.394	3.362	3.330	3.279	3.130	3.027	0.093
B	3.343	3.314	3.266	3.205	3.044	2.795	0.138
C	3.253	3.228	3.217	3.132	2.915	2.677	0.112
D	2.952	2.981	2.897	2.724	2.483	2.104	0.091
E	2.422	2.361	2.241	2.190	1.874	1.512	0.086
F	1.821	1.781	1.644	1.469	1.281	0.982	0.090
G	1.201	1.239	1.070	0.951	0.828	0.639	0.080
H	0.095	0.122	0.114	0.112	0.115	0.089	0.063

Table 20: ELISA plate serial dilutions for coating of F18 antigenic protein (*µγ/mL**ng/mL).

100 mg of seeds were homogenized in extraction buffer (TRIS-HCl, pH 7.5) then centrifuged, from the supernatant. The extracted proteins were estimated about 1.8 µg / µL (Bradford assay). 100 µl of each of the five standard and 100 mL of each sample were added into tubes. 100 µl of primary antibody was added, diluted in PBS buffer 1:12000. The samples were leaved an hour at RT. 100 µl was transferred of the previous competition reaction in the wells of the strip

ELISA (Coating 0.5 µg / mL) for an hour at RT. Then, the wells were washed three times with 1X PBS buffer. The secondary antibody (1: 15,000 in 1X PBS) was added and incubated for 30 minutes at RT. After washing the plates, 50 µl of TMB were added to each well and manteined in the dark for 15 minutes. The reaction was stopped with HCl 0.4 N and the optical density was measured at 450 nm.

3.5.5-Quantification of VT2e-B in vaccinal seeds

The coating of antigen was performed overnight (o/n) at 4°C in carbonate buffer (50mM, pH 9.6). Serial dilutions, starting from 10 µg/mL to 10 ng/mL, were tested starting from line 1 until line 12 as shown in the table 21; line 7 is the negative control (without antigen).

Different dilutions of the primary antibody starting from a 1:400 dilutions up to a 1.25000 dilution (line A-G) (line H negative control) without antibody were tested (Table 21). The conditions of the secondary antibody (anti-rabbit peroxidase conjugate) were fixed, dilution 1: 15000, as suggested by the manufacturer (Sigma).

Cal	1 10*	2 5*	3 2.5*	4 1.2*	5 0.6^{2*}	6 0.3^{1*}	7 0.15*	8 0.07*	9 0.03	10 0.019*	11 0.009*	12 10**
A	3.27	3.39	3.37	3.26	3.27	3.28	3.14	3.04	2.91	2.67	1.661	0.821
B	3.16	2.97	3.10	3.15	3.11	3.03	2.94	2.88	2.62	1.96	1.67	0.81
C	2.92	2.60	2.64	2.51	2.85	2.59	2.65	2.42	1.93	1.60	1.27	0.58
D	2.58	2.05	1.97	2.07	2.08	1.71	1.77	1.842	1.697	1.29	1.13	0.39
E	1.55	1.37	1.43	1.42	1.49	1.41	1.09	1.028	0.974	0.801	0.705	0.28
F	1.14	1.06	1.039	1.12	1.06	0.84	0.71	1.03	0.71	0.49	0.44	0.23
G	0.78	0.75	0.78	0.83	*0.68*	0.67	0.61	0.61	4.46	0.33	0.34	0.22
H	0.53	0.49	0.53	0.71	0.62	0.46	0.45	0.49	0.45	0.49	0.31	0.25

Table 21: Serial dilutions for coating of VT2e-B protein *: µg/mL**:ng/mL.

The best concentration for the coating was 0.6 µg/mL-10ng/mL and 1:6000 for the primary antibody. Based on the yield putatively contained in seed, five standards were determined from 0 to 80 ppm. 100 mg of seeds were homogenized in an extraction buffer (TRIS-HCl, pH 7.5). After centrifugation (5,000 rpm for 10 minutes), the supernatant was collected. The total extracted proteins were about 1.8 µg/mL (Bradford assay). The samples were finally quantified. 100 µl of each of the five standard and 100 µl of sample were putted into tubes. 100 µl of primary antibody (1:12,000 PBS buffer) were added for one hour at RT. 100 µl of the previous competition reaction were added in the wells

of the strip ELISA (coating 0.6 μg/mL) for an hour at RT. The samples were washed with 1X PBS buffer. The secondary antibody (1:6,000 PBS) was added and incubated for 30 minutes. 50 μl of TMB were added into tubes for 15 minutes without lightning. The reaction was blocked with HCl 0.4 N and the optical density was evaluated at 450 nm. The samples were analyzed with ELISA device at a wavelength of 450 nm, the calibration curve was calculated with the "Curve expert 1.3" program.

3.6-Microscopical evaluation of plants

We have performed analysis by optical and electronic microscopes in different phases of the plants growth in collaboration with the Department of Biosciences, Università degli Studi di Milano. Samples were fixed in 2% formaldehyde and 0,2% glutaraldehyde (in Hepes 50mM, pH 7,4) overnight at 4°C, repeatedly rinsed in Hepes 50mM, pH 7.4, dehydrated with increasing concentrations of ethanol and embedded in LRGold resin at -20°C.

3.7-In-vivo trial: evaluation of the oral administration of tobacco seed as edible vaccines in weaned piglets

3.7.1-Animals, Housing conditions

Thirty-six piglets (Landrace x Large White) were selected from a conventional herd free from diseases according to the A-list of the International Office of Epizootic, and from Aujeszky's disease, atrophic rhinitis, transmissible gastroenteritis, porcine reproductive and respiratory syndrome and salmonellosis, without history of PWD and OD and with bacteriological analysis of the feces negative for *hemolytic E. coli* (IZSLER).

Piglets, homogeneous per weight, age and gender (50% male, 50% female), weaned at 21 days (to ensure the animals' sensitivity upon experimental infection) were transported to the Experimental Animal Research and Application Centre (pigs sector) in Lodi of the University of Milan.

Animals were allocated in individual pen, under the same environmental conditions (temperature 29 °C and humidity 60 %), with water and feed *ad libitum*, the animals were identified by auricular tag. The *in vivo* trial was approved by ethical authorities (102/2015 PR Health Ministry) in accordance

with the European regulations (European Union Directive 2010/63/UE). The piglets were fed a basal diet without antibiotics, provided by Ferraroni S.p.A (Bonemerse CR, Italy) (Table 22).

The experimental diets were analyzed for the principal chemical components: crude protein (CP), according to the official method of Analysis of Association of Analytical Communities, procedure 2001.11 (AOAC, 2005); dry matter (dm), according to procedure 930.15 (AOAC, 2005); fat (EE) according to DM 21/12/1998; crude fiber (CF), according to procedure Ba 6a-05 of the official method of the American Oil Chemists Society (AOCS, 1998); ash, according to the procedure 942.05 (AOAC, 2005).

The animals were divided in two experimental groups, controls group (CG; n=18) and treated group (TG; n=18) randomly.

Item	Basal diet	High protein diet
Ingredient, g/kg		
Barley	228.000	161.880
Wheat flakes	168.000	119.280
Wheat meal	134.000	95.140
Maize flakes	112.000	79.520
Barley flakes	72.600	51.546
Soy protein concentrate	70.000	49.70
Soybean meal	-	28.000
Whey	55.600	39.476
Maize meal	52.000	36.920
Fish meal (herring)	39.700	28.187
Monohydrate dextrose	37.100	26.341
Spray-dried plasma	27.100	19.241
Coconut oil	27.000	19.170
Soybean oil	16.000	11.360
Dicalcium phosphate	5.200	3.692
Calcium carbonate	1.100	0.781
Sodium butyrate 30%[1]	2.190	1.555
L-Lys	6.700	4.757
DL-Met	3.220	2.286
L-Thr	3.040	2.158
L-Trp	1.220	0.866
Vitamin/mineral premix [2]	3.270	2.322
Vitamin E 50%	0.140	0.099
Additives: phytase[3], xylanase[4], acidifiers[5], feed flavors.	16.55	11.750
Chemical composition		
DM	90,332	88.5
CP	17,80	25,42
Fat	5,87	4,57
Crude Fiber	2,19	3,20
Ash	4,46	5,17
Starch + sugar	51,51	38,88
Lysin	1,48	1,73
NE Mc/Kg	2,60	3,56

Table 22: Basal diet and high proteins diet. [1]Palm oil, salts of fatty acids (sodium butyrate 30%), calcium carbonate. [2]Providing the following nutrients (per kg of air-dried diet): Vitamin A, (E 672) 4000000 UI/Kg; Vitamin D3, (E 671) 400000 UI/Kg; Vitamin E, (3a700) 40000 mg/Kg; Vitamin B1, 1200 mg/Kg; Vitamin B2, 4000 mg/Kg; Calcium D-pantothenate, (3a841) 10865 mg/Kg; Vitamin B6, (3a831) 2400 mg/Kg; Vitamin B12, 16 mg/Kg; niacinamide, (3a315) 14000 mg/Kg; Vitamin K3, 2000 mg/Kg; Folic acid, (3a316) 600 mg/Kg; D-Biotin, 80 mg/Kg; Choline chloride, (3a890) 90000 mg/Kg, Fe (FeO), 45000 mg/Kg; Cu ($CuSO_4$), 8000 mg/Kg; Zn (ZnO), 52200 mg/Kg; Mn (MnO), 16000 mg/Kg; I ($Ca(IO_3)_2$), 240 mg/Kg; Se (Na_2SeO_3), 120 mg/Kg. [3]Phytase (EC 3.1.3.26) minimum: 10000 Fytα-phytase/g [4]Endo-1,4β-Xylanase (IUB/EC 3.2.1.8) minimum 1000 FXU/g.[5] Ortophosphoric acid 33.5%, Calcium formate 32.38%, Citric acid 7.8%, Fumaric Acid 5%, Silicic acid 6%.

3.7.2-Experimental design

The work was divided in three phases:

1. Phase I-Immunization (Days 0-20): administration of vaccinal seeds by oral route (day 0, 1, 2, 5, 14).
2. Phase II -Challenge (Day 20): experimental infection.
3. Phase III- Observation (Days 21-29): sample collection, zootecnical and clinical evaluation

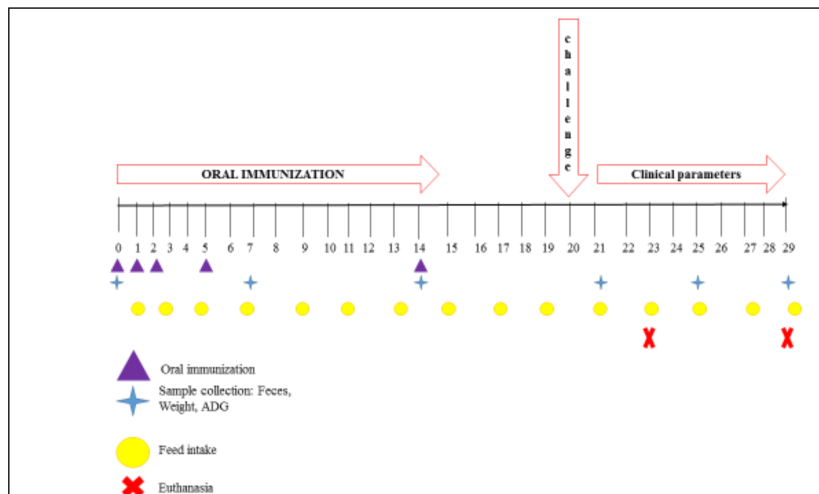


Figure 10: Scheme of experimental design.

3.7.3-Treatments

Tobacco seeds were ground with a suitable mill to obtain a uniform powder using a 0.75 mm grid (Figure 11) controlling the temperature (max 50° C).

Figure 11: ZM 200 Ultra Centrifugal Mill, Retsch® for fine grinding of *N. tabacum* seeds**.**

During the immunization phase (on days 0, 1, 2, 5, 14), the tobacco seeds were administered with a commercial milk replaced powder (LACTOSOL) by mixing

in a 1:2 ratio (weight/weight) in order to guarantee the complete consumption (Table 23).

Analytical components	Nutritional additives (/kg)
Crude protein: 20.5% Oils and fats: 20.0% Crude ash: 9.5% Crude fiber: 0.1% Sodium: 0.8% Lysine: 1.7% Methionine: 0.8%	Vitamin A (E672): 50.000 I.U. Vitamin D3 (E671): 5.000 I.U. 3a700 Vitamin E (all-rac-alpha-tocopher l acetate): 100 mg Vitamin C (E300): 100 mg E1 Ferrous sulphate monohydrate: 304 mg E2 Anhydrous calcium iodate: 1.5 mg E4 Copper sulphate pentahydrate: 31 mg E5 Manganous sulphate monohydrate: 169 mg E6 Zinc sulphate monohydrate: 192 mg E8 Sodium selenite: 0.55 mg

Table 23: Chemical component of LACTOSOL TV, used for the treatment preparation.

The TG received vaccinal *Nicotiana tabacum* seeds (F18s + VT2e-Bs) on days 0, 1, 2, 5 and 14. In particular, each treatment was represented by 10g of F18s (containing about 7 µg of FedA protein), 10g of VT2e-B (containing about, 35 µg of VT2e-B) and 40g of milk replacer powder. At the same time CG received 20g wild type milled tobacco seeds mixed with 40g of milk powder (Table 24).

Name of group	Number of animals	Treatment	Dose
Treated group (CG)	18	10 grams F18 and 10 grams VT2e-B 20 grams of milk powder	7 µg F18 35 µg VT2e-B
Control group (TG)	18	20 grams of WT milled tobacco seeds 20 grams powder milk	0 g

Table 24: Treatments received during the trial by the experimental groups.

3.7.4-Challenge

Six days after booster immunization, on day 20th, 24 piglets (TG, n: 12; CG, n: 12) were orally challenged with O138 *Escherichia coli* strain, provided by Lombardia AND Emilia Romagna Experimental Zootechnic Institute (IZSLER) (Table 25).

Days	Control Group (CG)		Treated group (TG)	
0-20 (Phase I)	N= 18		N= 18	
20-29 (Phase III)	Challenge group (CC)	Unchallenge group (UC)	Challenge group (CT)	Unchallenge group (UT)
	N= 12	N= 6	N= 12	N= 6

Table 25: Distribution of piglets in the experimental groups during the phase I and III.

Sixty minutes before challenge, piglets were sedated with azaperon (StresnilTM, Janssen Cilag SpA, 2 ml/head), thereafter 30 mL of a 10% bicarbonate solution (SIGMA, Italy) was orally administered in the attempt to neutralize gastric acid and to increase the survival rate of challenger strain in the stomach (Madec et al., 2000, Jensen et al., 2006).

After 10-15 min, the inoculum was given via oral route in a single dose of 5 mL of bacterial medium with $1*10^{10}$ colony forming units (CFU) of challenger strain, using 16G catheter. Animals were fasted 3 hours before and 3 hours after challenge.

For each group, six animals were not challenged and housed in pens separated by means of two empty pens from the infected group, so that physical contact between challenged and not challenged piglets was excluded.

From the day of challenge until the second day after challenge, the same antimicrobial-free diet, containing 27% of crude protein on dry matter, was administered to all experimental groups.

Before the administration, chemical analysis of the diet was performed to confirm its high protein level and to measure its principal components: crude protein (CP), according to the official method of Analysis of Association of Analytical Communities, procedure 2001.11 (AOAC, 2005); dry matter (dm), according to procedure 930.15 (AOAC, 2005); fat (EE) according to DM 21/12/1998; crude fibre (CF), according to procedure Ba 6a- 05 of the official method of the American Oil Chemists Society (AOCS, 1998); ash, according to procedure 942.05 (AOAC, 2005).

3.7.5-Sample collection and zootecnical evaluation

Blood samples were collected from the jugular vein of each animal to determine the hematocrit value on days 0, 7, 14, 21, 25, and 29.

In phase I, fecal samples were taken from rectum weekly on 0, 7, 14, 21 days to determine total and specific IgA levels, as described below.

In phase III fecal samples were collected in order to evaluate the shedding of the challenger strains in collaboration with IZSLER.

Rectal temperature was daily recorded and the clinical signs of the disease in particular: palpebral Edema, epiphora, vitality, respiratory and neurological problems - were daily checked and scored using the specific point scales reported below:

Respiratory score: 0 = normal; 1=slightly quick; 2= quick.

Edema score in palpebra: 0= normal; 1=mild; 2=severe.

Epiphora score: 0= normal; 1=mild; 2=severe.

Vitality score: 0= good; 1=loose; 2=bad.

In the same period, fecal consistency was daily evaluated through a scale of four levels: 0= normal feces; 1=soft consistency 2=mild diarrhea; 3=severe diarrhea. All piglets were individually weighed weekly during the entire experimental period and twice in the week following challenge (25 and 29 days); the feed intake (FI) was daily measured weighing the residual feed at the pen level (experimental unit for FI evaluation).

At day 3 post-challenge (23th day), 12 piglets (4 CC, 2 CU 4 TC, 2 TU) were euthanized for post mortem examination; the restraint part of animals were sacrificed at 29th day.

3.8-Evaluation of intestinal Immunoglobulin-A titer in the immunization phase

After the extraction of fecal antibodies, the titre of total IgA in fecal samples was evaluated through the Pig ELISA quantitation set (Bethyl Laboratories, Inc. Montgomery, Texas). Briefly, 0.5 g of feces were diluted in 1 mL of extraction buffer and placed for 1 hour in ice. Centrifuged at 2000*xg* for 15 min at 4°C. The supernatant was collected and further centrifuged at 10000*xg* for 10 min at 4°C. The supernatant was then removed and stored at 20°C until use.

3.8.1-Evaluation of total immunoglobulin A in the immunization phase

"Pig ELISA IgA quantitation set" was performed to detect and quantify IgA fecal samples. Fecal samples were diluted 1:100 for quantification of IgA. Serum

samples were diluted 1:100 in Sample Diluent, ("ELISA quantitation set", Bethyl Laboratories, Texas).

Microtitration 96-well plates were coated for 1 h at RT with 100µl of a solution containing 1 µl of affinity purified Pig IgA diluted in 100 µl of coating buffer. Then the wells were blocked with 200 µl of Blocking Solution for 30 min and then washed five times with 300 µl of Wash Solution. To each well 100 µl of standard or sample were added and incubated at RT for 1 hour. After washing the plates five times, 100 µl of diluted HRP detection antibody were added to each well and incubated at RT for 1 hour. After washing the plate five times, 100 µl of TMB Substrate Solution were added to each well. The plate was developed in the dark at room temperature for 15 minutes, and then reaction was stopped by adding 100 µl of Stop Solution to each well. Absorbance was measured on a plate reader at 450 nm. For each plate, a standard curve was constructed to calculate the IgAs concentration of each sample using "Curva Expert 1.3" software. The concentrations determined were expressed as nanograms of IgAs per 1 mL.

3.8.2-Evaluation of anti-F18 IgAs and anti-VT2e-B IgAs in fecal samples

An ELISA method was set up for the titration of specific IgAs for antigenic proteins.

In order to establish specific IgA title (anti-F18 and anti-VT2e-B) previously obtained antigens and antibodies were used.

<u>Extraction of total fecal immunoglobulins</u>

1g of fresh feces were vortexed (in 10 mL of extraction buffer 0.01M PBS, 0.5% Tween, 0.05% sodium azide) and centrifuge at 1,500 *xg* for 20 min at 4°C.

2 mL of the supernatant were transferred to a sterile tube containing 20 µL of protease inhibitor (Roche) and mixed briefly. Finally, the samples were centrifuged at 10,000*xg* for 10 min and the supernatant was recovered in sterile tubes.

For the evaluation of specific IgA (anti-F18 and anti-VT2e-B) a double antibody sandwich ELISA system was developed.

Pig IgA antibody (Bethyl 1: 500 in coating buffer, 50 mM sodium carbonate pH 9.6) is made to adhere to an ELISA plate o/n at 4°C. The plates are "blocked" with PBSt and 3% BSA to 3 hours at RT. The total immunoglobulins extracted from feces and/or their dilutions (100 mL) were incubated o/n at 4 ° C in the wells with the antibody conjugates. After three washes of the wells with PBSt, 100 μL are added to each well and incubated 1 hour at RT, respectively Ab F18 and Ab VT2e-B (Rabbit). After three washes of the wells with PBST, 100 μL of anti-Rabbit horseradish peroxidase conjugate were added to each well for 1 hour at RT. TMB was used for the colorimetric reaction; the quantification of the specific antibodies was out by measuring absorbance at 450 nm.

3.9-Statistics

Data were analyzed using IBM SPSS Software (IBM Corporation 1 New Orchard Road Armonk, New York 10504-1722 United States). We have applied Non-Parametric Tests for Independent Samples, using NPTESTS procedure post hoc tests for the Kruskal-Wallis omnibus test. Comparisons of antibody titers between groups were done using the Mann–Whitney test after base 10 logarithmic transformation. A p-*value*< 0.05 was considered to indicate statistical significance. In addition, the *one-way* analysis of variance (ANOVA) with a Dunnett post hoc test in SPSS. The Dunnett test is used when only the comparisons between treatments and a control group are of interest. Differences between groups were considered significant with a p <0.05. GLM univariate with Bonferroni *post-hoc* test.

4-RESULTS AND DISCUSSION

The health status of food-producing animals is a major challenge on a global basis as it affects upon both human health and economics, being a primary factor in determining safety, quality and health benefits of food of animal origin. Indeed, infectious diseases can spread rapidly with devastating results. Traditionally, the control of animal diseases relied upon the use of in-feed antibiotics, which have been used in animal production for over 50 years as feed additives. However, the use of antibiotics has raised increasing concerns about the development of antibiotic resistant bacterial strains, which may impair the effectiveness of certain antibiotics in the treatment of human diseases. In the last decennium, several innovative approaches were studied and developed. Animal nutrition, which for long time was based on the need to avoid deficiency symptoms and to support the economically important production criterion, in recent years has driven the research of new strategies for disease prevention, and a better understanding of the interactions between nutrition and health introduced the concept of nutrition-based health for animal production (Adams, 2006). Considering the pig livestock, various nutritional approaches have been tested in the past decades in order to manage critical periods in the lifespan of the animal, such as the weaning period of the piglet when the animal is more exposed to diseases (Kraeling and Webel, 2015; Moehn et al., 2013).

In general, it is always preferable to prevent disease rather than having to resort to treatment for animals: "Prevention is better than cure".

The main theme of this research concerns the nutritional management of some health problems frequently occurring during the weaning of piglets, focusing in particular on the use of tobacco seeds as edible vaccine against verocytotoxic *E. coli.* In swine livestock, the weaning is a particularly complex period, during which the piglets are confronted by multiple stressors. The immediate effect of weaning is a dramatic reduction in feed intake and a consequent 'growth check', which continues to represent a major source of production losses in commercial piggeries. Weaning also causes alterations in intestinal architecture and function, predisposing to diarrhea and intestinal infections. Verocytotoxic *E. coli* strains,

in particular O138, O139 and O141 serotypes, are an important causes of growth retardation and mortality in piglets during the first weeks after weaning.

Verotoxin-producing *E. coli* (VTEC) also presents adhesive fimbriae, important virulence factor, that is responsible for the adhesion of the bacteria on the intestinal wall.

The weaning process increases the stress on piglets, and thus their susceptibility to viral and bacterial infections. Up to now, no vaccine for post weaning diarrhea-protecting piglets against these infections is available and treatments rely upon the use of antimicrobials. Vaccination of the sow can enhance the protection and so far, several maternal vaccines are available on the market (Cox et al., 2002). Available commercial vaccines against swine *E. coli* infection are referred to colibacilosis that differs from post weaning diarrhea, because maternal immunity can be induced by vaccination and can result in the animals protection.

In recent times, the development of vaccines has rapidly advanced thanks to a significant progress made in the use of recombinant gene technologies. Plants have been recognized as an expression system for the production of edible vaccines thanks to the possibility of introducing antigenic proteins into their genome. Stable transformation allows transgene integration into the plant genetic material and, consequently, the transfer of the acquired character to next generations.

Delivery of a vaccine to a mucosal surface induces a mucosal immune response, providing a first line of defense against infection.

Several orally delivered vaccine candidates induced mucosal immune response in the gastrointestinal tract, such as Dukoral by (SBLvaccine), against cholera and Vivotif against rotavirus produced by (SSVI, Berna). However, the most promising targets for plant-based oral vaccines may be gastrointestinal pathogens (Sirskyj et al., 2016; Banda et al., 2012).

Many antigens from viral and bacterial origin has been expressed in plants against different animals diseases including polio, plague, cholera, malaria, canine parvovirus, tuberculosis, antrax, rotavirus (Arlen et al., 2008; Gorantala et al., 2014; Lentz et al., 2010; Ortigosa et al., 2010 Shao et al., 2008).

The work presented in this thesis is part of a wider project, still in progress in our Department, in which *Nicotiana tabacum* plants were previously engineered for the seed-specific expression of the major subunit FedA of the F18 adhesive fimbriae, and VT2e B-subunit genes respectively, as model of edible vaccines against porcine verocytotoxic *E. coli* (Rossi et al., 2013). The major subunit FedA, the primary pathogenic factor responsible for the adhesion of *E. coli* strains to enterocyte receptors, and the B subunit of the toxin VT2e were chosen as antigens. Specifically, the development of toxicity is caused by the extra-intestinal effects of VT2e toxins, which are bipartite molecules, composed of a single enzymatic intracellularly active A-subunit and a pentamer of B-subunits associated with receptor binding.

In the present study, two lines of *Nicotiana tabacum* plants were previously transformed via *Agrobacterium tumefaciens* with chimeric constructs containing structural parts of genes under control of a seed specific GLOB promoter.

In the first part, the attention was focalized on the production of tobacco seed-based oral vaccines against verocitotoxic *E. coli*. Then the vaccinal seeds were *in-vivo* evaluated through a challenge experiment on weaned piglets.

4.1-Production of tobacco seed-based oral vaccines against verocytotoxic Escherichia coli production

4.1.1-Cultivation and collection of seeds

The development of vaccines has rapidly advanced thanks to a significant progress made in the use of recombinant gene technologies. Plants have been recognized as an expression system for the production of edible vaccines thanks to the possibility of introducing antigenic proteins into their genome. Stable transformation allows transgene integration into the plant genetic material and, consequently, the transfer of the acquired character to next generations.

Transgenic plants would also permit large scale, low-cost expression of selected genes and have the potential for crossing transgenic lines to obtain multiple proteins production in the same plant. Moreover, they are able to confer heat stability to the heterologous protein and, if edible plants are used, the antigen can be attractively delivered through oral administration in feed, thus avoiding

antigen purification and needle administration (Tregoning et al., 2004). Furthermore, plants or plant products, which are edible, provide the additional unique opportunity of serving as vehicles for oral delivery of the vaccine.

Tobacco is a prolific seed producer, about 1 million seed/plant. In agricultural practices the production of tobacco seeds is 30-500g/ha, (10000 seeds/gram) and 140-150T/ha of total biomass (Fogher et al., 2007).

Nicotiana tabacum is an organism model and extremely versatile system for all aspects of cell and tissue cultures research. Tobacco is a natural allotetraploid formed through two hybridization between two diploid progenitors, *Nicotiana syslvestris* and *Nicotiana tamentasiformis* approximately 6 million years ago. Based on some issues the optimization of cultivation practices of tobacco plants had been studied for the purpose of obtaining the maximal seed production with high levels of stable transgenes.

SEED	Germination (%)
WT	78.5
F18	73.3
VT2e-B	76,6

Table 26: Germination index of tobacco plants.

We had harvested the third generation (R_3) of two lines of *Nicotiana tabacum* expressing, respectively, adhesive fimbriae FedA and VT2e-B toxin (VT2e-B) in order to obtain an effective quantity to perform an *in-vivo* trial. Three lines of seeds, two vaccinal and one wild type tobacco, were potted in each pots of seedbed. No statistical difference was observed in germination index between the *Nicotiana tabacum* control and engineered tobacco seeds (Table 26).

We had 80 pots for vaccinal tobacco lines and 56 pots for wild type tobacco plants grown in a Fitotron® growth chamber. This is in according to the observations about plants germination. 80% germination rate, estimating losing about 20% during transplanting and even taking in a 10% mortality rate for first time growers (source: Seedman .com).

After 5 weeks from seeding wild type, plants showed a higher height if compared to the vaccinal lines (Figure 12). No differences were observed between F18 positive plants and VT2e-B positive plants.

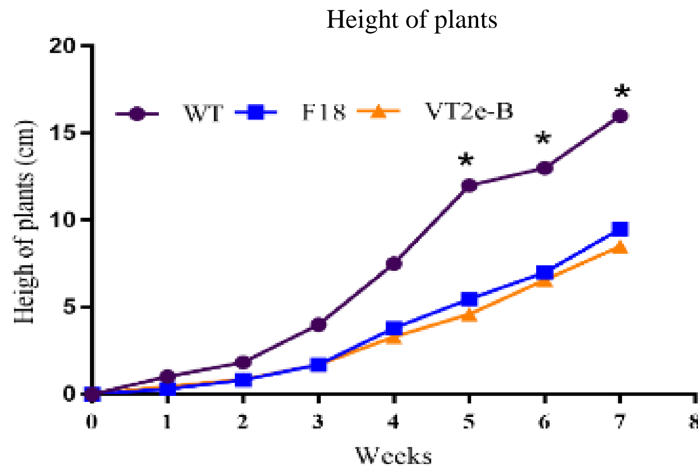


Figure 12: Growth curves of *Nicotiana tabacum* plants after the seeding (day 0); * *p-value:* <0.05. WT, n:56; F18, n:80; VT2e-B, n:80.

The data were analyzed by the univariate GLM procedure with estimated marginal means and with the Bonferroni *post hoc* test, profile plots and custom hypothesis tests showed that the heights of engineered plants (F18 and VT2e-B) and the heights of the control plants did not show statistical differences at T_0, T_1, T_2, T_3 T_4, (*p-value* > 0,05). The heights of the control plants compared to height of transgenic plants (F18 and VT2e-B), instead, was statistical different at T_5, T_6, T_7 (*p-value<0.05*) but the heights of F18 compared with the heights of VT2e-B did not show statistical difference (*p-value* = 0,208) during the growth period.

Considering the day of seeding as day 0, wild type lines germinated at day 6 and the two vaccinal plants at day 10 (Figure 13-14)

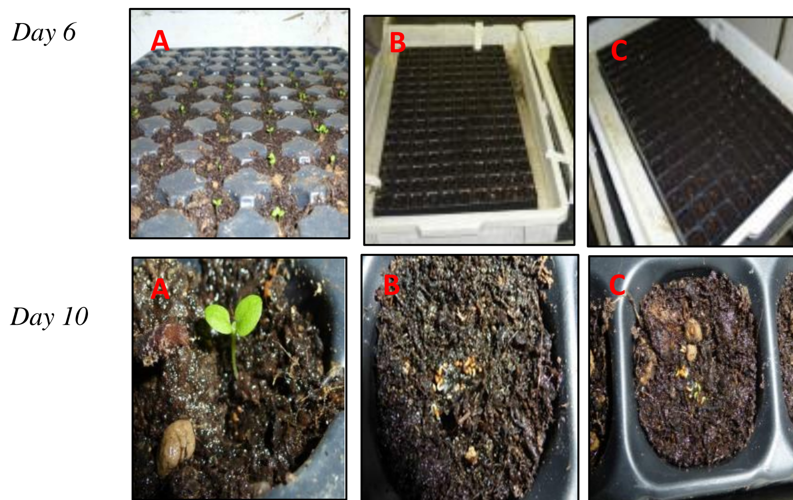


Figure 13: Images representing the beginning of the growth phases of the experimental plants at day 6 and at day 10. A: wild type plants; B: VT2e-B; C: F18 plants.

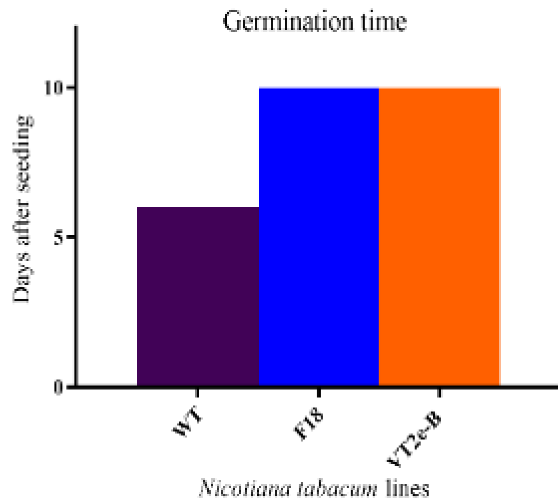


Figure 14: Germination time (days after seeding) of wild type and vaccinal tobacco lines.

Nevertheless, F18s and VT2eBs germinated four days later than wild type, as showed in Figure 14. This aspect was probably caused by late metabolic employment of inclusion bodies, observed in the vaccinal seeds by morphological analyses described below.

When plants were about 15 cm high, 80 plants for each transgenic line were transferred to the "Orto Botanico G.E. Ghirardi" in Toscolano Maderno (Brescia). The plants were grown in accordance to a Directive which governs (Directive 98/81/EC of 26 October 1998, amending council directive 90/219/EEC), on the contained use of genetically modified organisms. The specific biosafety level of the greenhouse used was level 1.

Transgenic plants are categorized in this level when both host plant and transgenic plant pose little risks for humans, animals, plants or the environment. "Environment" indicates the environment, which is likely to be exposed during the planned activities. Within the containment of a greenhouse, the exposed environment is very limited.

The greenhouse was previously supplied with polyethylene monofilament that provides protection against aphids, white fly and *bemicia tabaci* and lamp to guarantee a photoperiod of 14h light and 10h dark. The environmental conditions at the Orto Botanico G. E. Ghirardi were a temperature between 25-30°C for adequate growth and flowering, atmospheric humidity between 80 and 85% and a low level of nitrogen in the soil. The plants were treated with 2020-20 Peters

Professional® Water Soluble Fertilizer containing 20% of total nitrogen, 20% of Phosphate (P_2O_5) and 20% of Soluble Potash (K_2O). Also in the greenhouse, plants were stored isolated from the environment by the polyethylene monofilament to avoid cross-pollination.

The harvest of the vaccinal seeds occurred when the seeds pod turned brown. The harvest has been made from June to October. After harvest, seeds ovaries were stored in a cool, dry place to air dry for 7-10 days. After this period each ovary was opened in order to collect, transgenic seeds the harvest of seeds was performed in different rooms to avoid contamination between the two transgenic lines F18 and VT2e-B. Each ovary contains about 100 mg of seeds. Seeds were sift and separate from debris and the residual part of the ovary.

All 80 plants for each line produced good quality seeds without parasites. We performed some harvests and in total, we obtained 450 gr for 80 VT2e-B positive plants and 470 gr for 80 F18 positive plants. Each plant produced about 5-6 grams. Considering that each plants was cultivated in 20-25 cm^2 the production of seeds obtained during the trial was consistent with the space available in the greenhouse. In agricultural practices total biomass of tobacco yields on unirrigated land is usually 1.100-1.300 kg per four Km^2 and irrigated lands can produce about 2,000 kg per four Km^2 when using the closer commercial spacing of 0.5-1 meter between plants, with rows 1.2 meter apart (from Seedman.com).

The obtained yield of tobacco seeds was lower than commercial productions. It could be due to losses related to the manual harvesting. Nevertheless, it was sufficient for the further oral immunization trial.

4.2-Morphological evaluation of plants

The germination time between wild type tobacco plants and engineered tobacco plants was different. Transgenic tobacco plants germinated 4 days after the wild type. We have analyzed by optical and electronic microscope in different phases of plants growth in collaboration with the Department of Biosciences, Università degli Studi di Milano. The germination of transgenic seeds occurred four days later than the wild type in two replicated experiments, with 12 replicated. This suggested that the random integration of exogenous genes by *Agrobacterium* might influence genes related to

germination. For these reasons furthers morphological analyses were carried out. In Figure 15, a section of wild type plant embryo contained in seed is shown.

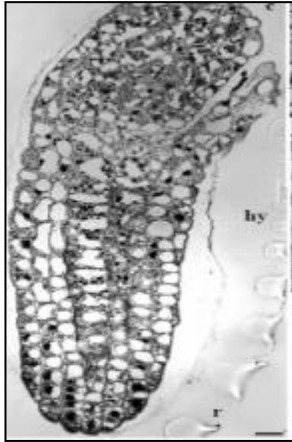


Figure 15: Ultra-thin sections (80 nm) of whole embryo of wild type tobacco plant, obtained using a Reichert Jung Ultracut E microtome. Semi-fine sections were stained by 1% toluidine blue and observed with a Leica DMRB optical microscope. C: cotyledon, hy: ipocotyledon, r: radicle. Magnification bar 50 µm

The F18 and VT2e-B engineered seeds (Figure 16) presented many storage bodies as protein bodies and lipid bodies that appeared as dark dots inside the cells by electronic microscopy.

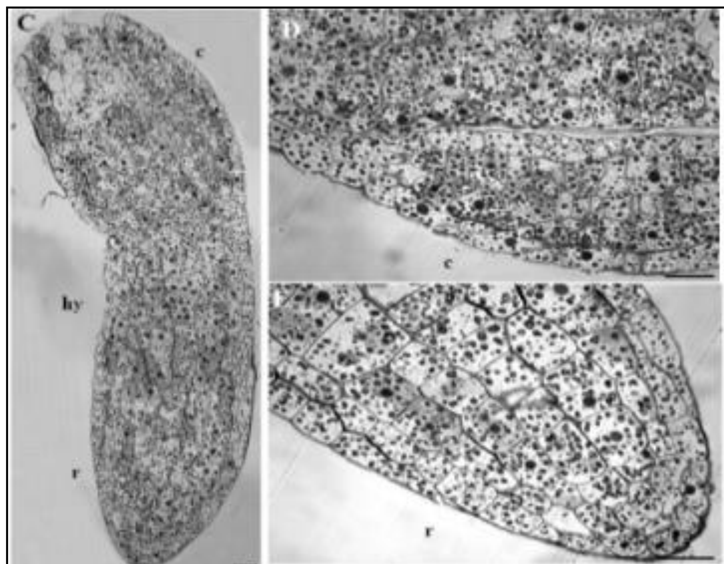


Figure 16: C-D-E.: Ultra-thin sections (80 nm) of whole embryo of F18 transgenic tobacco plant, obtained using a Reichert Jung Ultracut E microtome. Semi-fine sections were stained by 1% toluidine blue and observed with a Leica DMRB optical microscope. C: cotyledon, hy: ipocotyledon, r: radicle. Magnification bar 50 µm

Plant oleosomes are uniquely emulsified lipid reservoirs that serve as the primary energy source during seed germination. These oil bodies undergo significant changes regarding their size, composition and structure during normal seedling development. Oil bodies—filled with mostly triacylglycerols (TAGs) and to a lesser extent sterol ester, diacylglycerols, monoacylglycerols and free fatty acids (FFAs) are plant analogues to classical mammalian lipid droplets. They provide energy in the form of FFAs for β-oxidation in neighbouring glyoxysomes (peroxisomes) during initial seed germination and lipids for new cell and organelle membranes (D'Andrea, 2016). The structure of oil bodies also resembles lipid droplets. Owing to the initial absence of photosynthesis in germination, nearly all energy for initial development in plants comes from lipids via lipolysis of TAGs by surface-bound lipases, β-oxidation in glyoxysomes and catabolism in mitochondria (Graham, 2008). As a result, the density of oleosomes, and correspondingly of oleosins, was initially quite high: for instance, oleosins constitute nearly 10% of the total protein mass in *Arabidopsis thaliana* seeds (Hsieh, 2004). Although previous studies have shown that oleosin disappears from oil bodies during germination (Chen et al., 2014; Deruyffelaere et al., 2015) and that oil bodies fuse when oleosin is genetically suppressed (Siloto et al., 2006). In the same manner, plants store proteins in embryo and vegetative cells provide carbon, nitrogen, and sulfur resources for subsequent growth and development. The storage and mobilization cycles of amino acids that compose these proteins are critical to the life cycle of plants. Mechanisms for protein storage and mobilization serve many different developmental and physiological functions. For example, stored proteins provide building blocks for rapid growth upon seed and pollen germination. In the vaccinal plants, a late metabolic employment of inclusion bodies that is probably responsible for the delayed germination in the greenhouse was observed.

4.3-Chemical analyses of principal components of vaccinal tobacco seeds

Chemical analyses carried out on seeds from, F18 and VT2eB are reported in table 27. No differences were observed in the principal nutrient content. For these reasons, the vaccinal seeds could be considered substantially equivalent to the wild type.

Chemical composition % DM	F18 seeds	VT2e-B seeds
Crude protein	26.14	25.4
FAT	32.77	36.30
NDF	39.11	37.25
Ash	8.53	5.47

Table 27: Chemical composition of F18, VT2e-B *Nicotiana tabacum* seeds.

4.4-Molecular Analyses of tobacco plants

A pivotal aspect of this work was the detection of exogenous genes in the R_3 generation of tobacco plants in order to confirm the stable integration into the genome. The PCR products were evaluated on agarose gel (range 0.8-1.5%). PCR products of B-subunit of VT2e and the F18 fimbriae compared with the marker were respectively identified as bands with a length of 270bp and 519bp (Figure 17). The *NOS* gene was identified as a band with a length of 118bp (Figure 18).

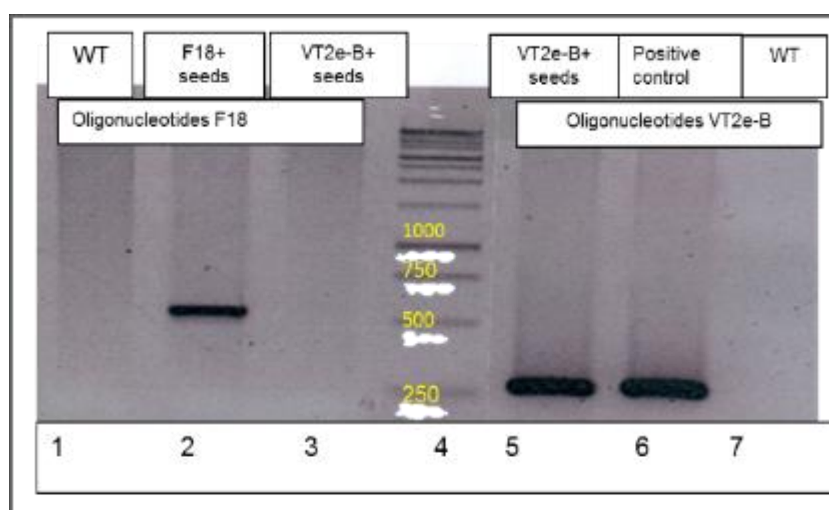


Figure 17: Agaraose gel of PCR products for the detection of VT2eB and FedA genes. Lanes 1, 2, 3, are represented by PCR products using oligonucleotides specific for F18 gene; Lanes 5, 6 7 are represented by PCR products using oligonucleotides specific for VT2e-B gene. Lane 1: Genomic DNA from wild type *Nicotiana tabacum*; Lane 2: genomic DNA from F18 positive *Nicotiana tabacum*; Lane 3: genomic DNA from VT2e-B positive *Nicotiana tabacum* plants; Lane 4: marker (bp); Lane 5: genomic DNA from positive VT2e B line 6: positive control. Line 7: WT.

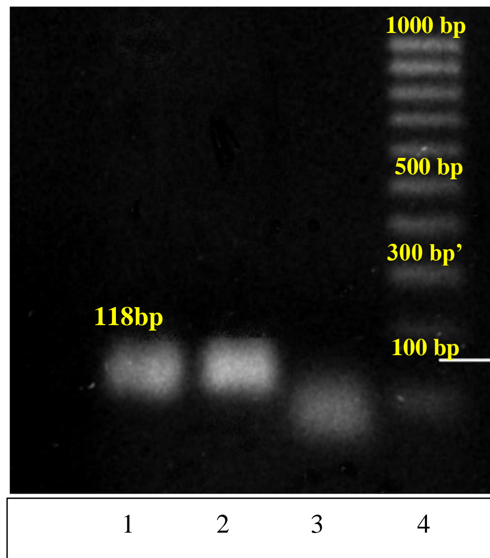


Figure 18: PCR for NOS *t*erminator (118 bp). Lane 1: DNA from VT2e-B positive tobacco seeds; lane 2: DNA from F18 positive tobacco seeds; lane 3: negative control; lane 4: marker 100bp.

The PCR of NOS gene terminator in our samples is a screening procedure used to assess that the exogenous gene of interest were integrated into the genome of cultivated tobacco plants. NOS gene derive from the nopaline synthase gene of *Agrobacterium tumefaciens* that is often used as an insert for genetically modified organisms (GMOs). PCRs were developed following the indications published in the JRC *"Compendium of reference methods for GMO analysis*" (2006).

Obtained data demonstrated that the genes coding for VT2e-B and the F18, representing important antigens and virulence factors of *E. coli*, could be stably incorporated into the next generation of *Nicotiana tabacum* genome (R_3).

4.5-Expression and quantification of antigenic proteins in tobacco seeds, by ELISA

We evaluated the expression of antigenic proteins in collaboration with PLANTECHNO S.R.L. The F18 and VT2e-B proteins were purified as a positive control.

ELISA assay to quantify antigenic protein F18 in transgenic tobacco line

Total protein extracted were estimated to be about 1.8 µg/ µL. The indirect ELISA assay was developed using TMB /HCl and the optical density was measured at 450 nm (Table 28).

Std µg/µλ	Lect.1	Lect.2	Lect.3	mean	dev std	cv %	interpol.
0	2.166	2.129	2.191	2.162	0.031	1.4	0.9
2	2.073	2.082	2.096	2.084	0.012	0.6	1.9
10	1.876	1.860	1.923	1.886	0.033	1.7	11.6
50	1.742	1.711	1.766	1.740	0.028	1.6	45.0
80	1.664	1.660	1.702	1.675	0.023	1.4	81.4
F18 TQ	1.987	1.875	1.945	1.936	0.057	2.9	7.4
F18 50%	2.004	2.050	2.013	2.022	0.024	1.2	3.3
WT TQ	2.196	2.200	2.234	2.210	0.021	0.9	0.6

Table 28: ELISA plate of FedA antigen. Output of lecture of program “Curva expert1.3” 450 nm. TQ: without dilution

The secondary anti-body molecule, which is covalently bound to horseradish peroxidase (anti rabbit H_2O_2–oxidoreductase), was incubated with developer (TMB). The developer contains both the substrate and the chromogenic reactants. When H_2O_2 is degrade, by-product causes the chromogen to develop a green color. According to the standard curve the amount of FedA subunit fimbriae was estimated to be about 66-74 ng per 100 mg of seeds (Figure 19).

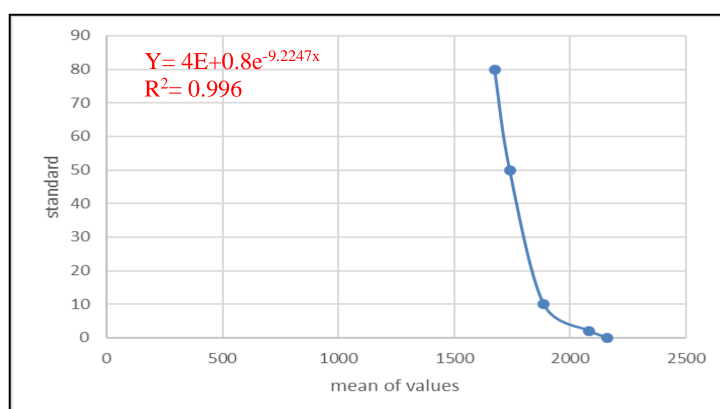


Figure 19: Standard curve performed by "curva express 1.3"program of FedA immunogenic protein in ELISA assay.

ELISA assay to quantify antigenic protein VT2e-B in transgenic tobacco line

In the same manner, we have evaluated VT2e-B, as results the best concentration for the coating was 0.6 mg/mL-10ng/mL and 1:6000 for the primary antibody. The samples were analyzed with an ELISA device at a wavelength of 450 nm, (Table 29) the calibration curve (Figure 20) was calculated with the program "Curve expert 1.3" The average content of VT2e-B in the 100 mg of seeds is equal to 340-370 ng of the immunogenic protein.

Std μg/μl	Lect.1	Lect.2	Lect.3	media	dev std	cv %	interpol.
0	1.445	1.560	1.461	1.489	0.062	4.2	0.5
2	1.320	1.393	1.283	1.332	0.056	4.2	2.2
1	1.260	1.157	1.164	1.194	0.058	4.8	8.6
50	1.029	1.039	0.977	1.015	0.033	3.3	50.9
80	0.976	1.002	0.918	0.965	0.043	4.5	83.3
VTe2-B	1.039	1.002	1.100	1.047	0.049	4.7	37.0
WT	1.586	1.551	1.577	1.577	0.023	1.4	0.2

Table 29: ELISA plate of VT2e-B antigen. Output of lecture of program "Curva Expert 1.3"at 450 nm.

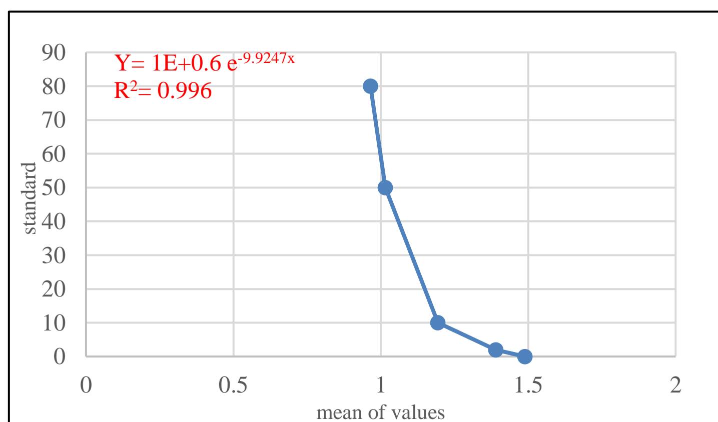


Figure 20: Standard curve performed by "Curva express 1.3" program for VT2e-B immunogenic protein in ELISA assay.

Moreover, we performed ELISA assays in order to assess that the nuclear apparatus can be able to translate the transgenes in antigenic proteins that were estimated to be around 6.6-7.4 μg and 34-37 μg per 10 g of seeds respectively for FedA and VT2e-B. This aspect is particularly important. Notably, during the transgene integration following *Agrobacterium*–mediated transformation, the

position of transgene integration is essentially random within the genome of the plant species. The transgene is thus subject to position effects which may influence its expression, resulting in some cases in transcriptional silencing as the new DNA is sequestered into the silenced region There are many factors that influence transgene stability, and these lead to highly variable expression within populations of plants generated in the same gene transfer experiment. One of the most important factors is the position effect, which reflects the influence of genomic DNA surrounding the site of transgene integration (Wilson et al., 1991). Another is the structure of the locus, including the number of transgene copies, their intactness, and their relative arrangement, which influences the likelihood of physical interactions and further recombination within the locus (physical instability) and the induction of silencing through DNA methylation and/or the production of aberrant RNA species from the locus (Heinrichs, 2008).

Based on these issues we assessed the production of vaccinal proteins by the nuclear apparatus of both tobacco plants lines. *Nicotiana tabacum* seeds offer the unique opportunity for the production and delivery of oral subunit vaccines. In several published papers, authors achieved a good expression of valuable proteins. Tackaberry (1999) produced human cytomegalovirus glycoproteinB (UL55) in seed of transgenic tobacco. Specific immunoassays of protein extracts from seeds of positive plants showed that all were producing antigenic glycoprotein B at levels ranging from 70-146 ng/mg extracted protein. Kohl and colleagues (2007) investigated the possibility and feasibility of producing the HPV-11 L1 major capsid protein in transgenic *Arabidopsis thaliana* ecotype Columbia and *Nicotiana tabacum* cv. *Xanthi* as potential sources for an inexpensive subunit vaccine. Yields of up to 12 μg/g of HPV-11 L1 NLS^- protein were harvested from transgenic *A. thaliana* plants, and 2 μg/g from *N. tabacum* plants.

The estimated amount of antigens allowed a further *in-vivo* trial.

4.6-In-vivo trial: evaluation of the oral administration of tobacco seed as edible vaccines in weaned piglets

4.6.1-Strategical optimization of vaccinal tobacco seeds administration

The most common uses of tobacco is for cigarettes; nevertheless, tobacco is covering important roles also in other holistic sectors and herbal medine.

Tobacco plays an important role in the advancement of plant biotechnology, it has been found to be an extremely versatile system for genetic manipulation and tissue culture research. Oral vaccination, through plant-derived immunogens, presents many potential advantages related to the management of intensive livestock, if compared with traditional injectable vaccination way. Considering the optimization of the oral immunization of piglets with tobacco seeds transformed for the expression of antigens against porcine verocytotoxic *E. coli* different aspects must be considered.

Firstly, the oral immunization through edible vaccines must be start at least at the weaning, because of simultaneous decreasing of maternal immunity and the exposure to high microbial pressure. During the weaning phase, piglets are exposed to several stress factors that may influence the establishment of the infections. The loss of the passive protection provided by milk, the rise in stomach pH, the slowed gut transit and the morphological and physiological changes in the small intestinal tract, which occur at weaning, allow bacterial adhesion and colonization. Stresses from mixing and moving into a new pen also cause increased transit time and depressed immune response through the release of cortisol. Predisposing factors to post-weaning infections include rearing conditions, in particular environmental temperatures, hygiene and dietary composition. Nutrition is obviously a critical determinant in the functional development and growth of the gastrointestinal tract and the weaning phase represents a critical period. The immediate effect of weaning is a dramatic reduction in feed intake and a consequent 'growth check', which could compromise the effective oral immunization. Effective needle-free immunization strategies are needed to

accommodate large-scale vaccination programs and avoid injection-related risks. Such delivery systems have broad applications for the rapid immunization of humans, as well as production farm animals, and may be the effective means for immunization of wild animal reservoirs to mitigate epizootic disease spread into humans and other animals. Vaccines administered at mucosal surfaces can induce local protection at the infection sites for many pathogens as well as induce systemic immunity (Ogra et al., 2001; Streatfield et al., 2006; Arntzen et al., 2003).

However, direct antigen delivery through an oral or nasal route generally leads to weak induction of immunity, if any, or to immunological tolerance. A previous study showed that tobacco seeds (*Nicotiana tabacum* specie) present less than 2 µg/kg of nicotine, a toxic alkaloid compound, and can be integrated in the feeding of the weaning phase of pig livestock without affecting the palatability (Rossi et al., 2007).

In order to ensure the complete consumption of the treatments in Rossi et al. (2013) piglets were fed tobacco seeds mixed with chocolate and water in palatable bolus. In this work, we chose to increase the palatability of tobacco seeds by adding a specific pig milk replaced powder (composed by dairy products, vegetable oils, cereals, sugars and flavoring). The milk replaced powder, based on protein content (about 20.5% as fed), was mixed with milled tobacco seeds in a 2:1 ratio (weight).

Moreover, a preliminary study showed that gastric degradation of milled transgenic tobacco seeds did not affect the protein signal by western blot analyses (Rossi et al., 2011). The ability of specific polyclonal rabbit serum to recognize the antigens even after 3 hours of incubation with porcine gastric fluid may indicate that the residual amount of transgenic proteins after digestion of both milled and whole seeds appears sufficient for their use in immunization trials on piglets.

Nevertheless, the seeds of *Nicotiana tabacum* are so small that 1 g seed contains 10,000-18,000 grains and present an impermeable coat. The grounding becomes pivotal for the formulation of the experimental feed, in order to guarantee the sufficient antigen exposure in intestinal mucosa. In

addition, tobacco has great potential as a vaccine delivery platform because of its naturally high protein content, nutritional value and multiple product streams. Mature seed contains about 35% (w/w) protein, primarily the 7S glycinin and 11S β-conclycinin storage proteins, compared to 8–10% protein in maize and rice or 1–2% protein in leaf tissues. Similar to other seed types, mature tobacco seed has a low water content that confers storage stability on expressed proteins.

Administration schedule of vaccinal seeds

To have a complete and mature immune response, the clonal expansion of B-cells must not only stop but an active process of cell death (apoptosis) must also occur. This whole process from vaccination to achieving mature immune response homeostasis takes at least 3 weeks. This fully developed mature immune response can then be boosted to get a true secondary response, typically swine vaccine primary and booster doses are administered at 2 weeks of interval. In young pigs, this is done to provide an opportunity to make sure that the piglets develop a primary response in the face of maternal immunity. Protection on mucosal surfaces is due in large part to secretory IgA, the route of vaccine administration can be important when attempting to induce mucosal immunity. To induce secretory IgA production at mucosal surfaces with a subunit vaccine, it is better to present the antigens at the mucosal level. We planned multiple oral administration in order to guarantee the booster effect at day 0, 1, 2, 5, 14.

4.6.2-Zootechnical performances

Piglets were early weaned at 21 days to ensure the sensitivity upon experimental infection. At the beginning of the experiment, the animals had homogeneous average individual body weight (Figure 21).

Since the start of experimentation during the first two days of housing, the animals were fed vaccinal tobacco seeds mixed with powdered milk (treatment). We have done it in order to guarantee the assumption of suitable antigenic dose by all enrolled subjects in sperimentation. The individual feed intake was measured from day 2nd through the weight of the residue in the feeder. The feed intake was reduced in the first days after weaning and then

gradually increase in all experimental groups, however no statistical differences were observed.

During the immunization period (days 0-20), after an initial phase of adaptation to the solid diet, in which feed intake and weight gains were low, the ADG and ADFI (Figure 22 and Table 30) were in line with the standards of growth of the piglets in this phase, with no statistically significant differences among the experimental groups. As shown by previous studies (Rossi et al., 2007), these data confirm that tobacco seeds intake and oral immunization had no effect on the zootechnical performances.

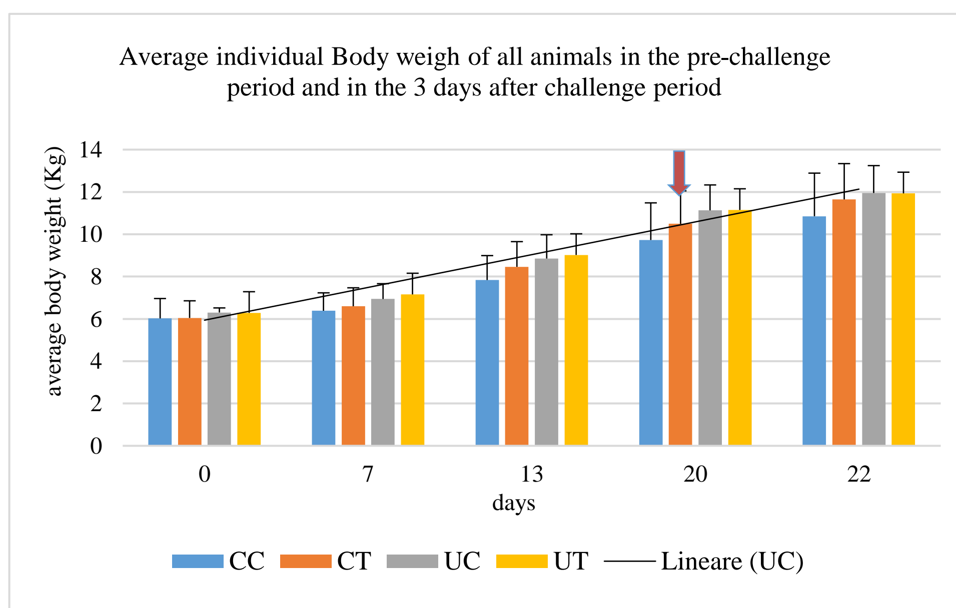


Figure 21: Evaluation of body in the experimental groups. Arrow represents the day of challenge

CC: challenged control, CT: challenged treatment, UC: unchallenged control, UT unchallenged treatment

Experimental groups	AFI in the pre-challenge period: day 0-20 (grams)
CC	328,6
CT	347,0
UC	367,0
UT	362,7

Table 30: Average individual FI in days 0-20. CC: challenged control, CT: challenged treatment, UC: unchallenged control, UT unchallenged treatment

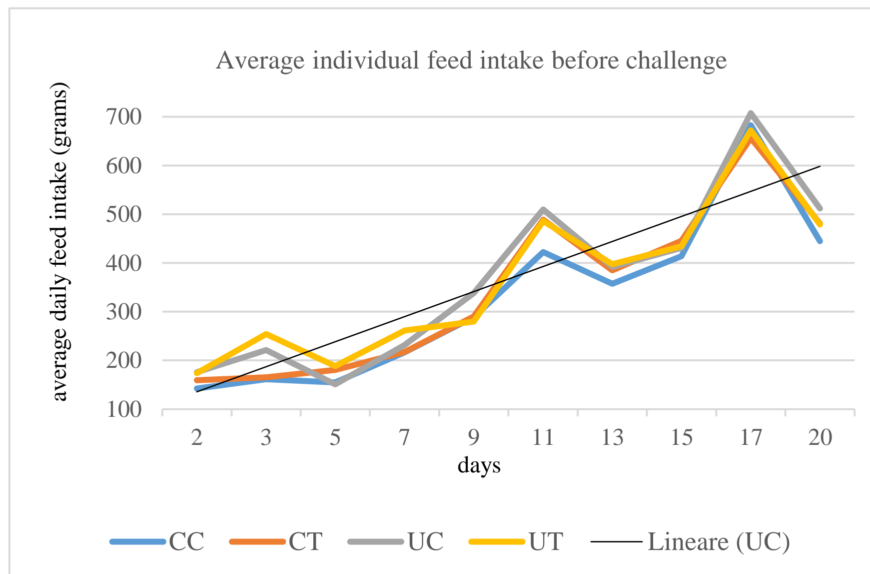


Figure 22: Average individual feed intake in the experimental groups during the pre-challenge. CC: challenged control, CT: challenged treatment, UC: unchallenged control, UT unchallenged treatment.

Since verocytotoxic *E. coli* is responsible for significant economic losses due to high mortality and reduction of growth rates of infected piglets, the zootechnical parameters were measured during the post-challenge period to assess whether oral immunization could reduce the negative impact of *E. coli* infection on the production parameters. As expected, the zootechnical parameters were clearly affected by experimental infection.

The three days after the challenge, a general reduction of feed intake was observed in all experimental groups. This is probably due to the high protein content diet, administered to all the animals; in fact, it could affect the palatability and create enteric disorders. The diet administered during the first three days, containing a high level of crude protein, was intended to be a "high-risk" diet, since several pathogens preferentially ferment proteins and high amounts of crude protein in the diet of newly weaned piglets have been identified as one of the predisposing factors of *E. coli* infection. Moreover, the high level of crude proteins was due to the presence in the feed of soybean meal, an ingredient that seems to allow the occurrence of *E. coli* infection (Fairbrother et al., 2005)

The experimental infection affected the daily feed intake, in fact challenged groups showed a significant lower feed intake if compare with non-challenged

groups. CC showed a lower AFI considering both periods after the challenge (Table 31).

Experimental groups	AFI days 17-20 (g) Before challenge	AFI day 21-23 (g) 3 days post-challenge	AFI days 20-29 Post-challenge period (g)
CC	563,8	456,9	482,1
CT	568,4	480,6	521,7
UC	609,6	530,8	568,5
UT	575,6	493,1	533,9

Table 31: Comparison of the average feed intake in the three days before challenge (17-20) and the periods after challenge (days 21-23; days 21-29). CC: challenged control, CT: challenged treatment, UC: unchallenged control, UT unchallenged treatment.

Challenge has certainly influenced the average daily gain (ADG) indeed the control challenge group and the treated challenge group showed a lower ADG during the completely experimental trial when compared with the ADG of uninfected groups. Nevertheless, considering only challenged groups, the treated challenge group showed a higher ADG than the control challenge group (Table 32).

	ADG 0-29 (g)
CC	227,34 ± 73,21
CT	243,31 ± 54,44
UC	271,96 ± 48,06
UT	264,47 ± 68,21

Table 32: Average daily gain in the completely experimental period. Values are expressed as grams ± standard deviation. CC: challenged control, CT: challenged treatment, UC: unchallenged control, UT unchallenged treatment

4.6.3-Evaluation of clinical signs

From the day after the challenge, clinical signs related to VTEC infection were observable in infected piglets. Clinical and fecal score related to VTEC infection, such as palpebral edema, epiphora, loss of vitality in all challenged groups from the day after challenge until day 9-post challenge are shown in table 33. For each clinical sign "the average total score" that is the sum of the average daily score of each group recorded from day 1 to day 9 post-challenge (Rossi et al., 2012) was considered.

ATS day 9					Significance		
	T		C				
	UT	CT	UC	CC	Treat	Chall	treat *chall
Edema	0.50	1.50	3.25	9.63	<.01	<.01	<.01
Epiphora	1.75	4.00	5.75	8.38	<.01	<.01	ns
Vitality	0.50	1	0	4.13	ns	<.01	ns
Hair	2.50	4.50	6.75	11.5	<.01	<.05	ns
Fecal score	3	7.13	9.00	11.6	<.05	Ns	ns

Table 33: Average daily score of experimental groups of animals at day 9. CC: challenged control, CT: challenged treatment, UC: unchallenged control, UT unchallenged treatment

What regards the palpebral edema, an important symptom related to the VTEC infection, CC showed a higher total score compared to UT, CT and UC ($p < 0.01$).

Considering the vitality, treated individuals groups showed a faster recovery after the challenge.

What regards the fecal score, during the nine post-challenge days, challenged control groups presented a higher average total score than the other experimental groups, without statistical differences. This aspect is probably due to the administration of the “high risk” diet during the three days after the challenge that could be responsible for enteric disorders as observed immediately after the challenge.

These results demonstrated that the piglets fed with tobacco seeds, which express the VTEC antigens, have generally a better clinical condition. This oral immunization strategy also seems effective to control the development of the clinical signs after the challenge with the O138 *E. coli* strain.

The use of the multicomponent vaccine appeared more effective in preventing the negative impact of challenge with O138 strain. This vaccination strategy probably provided a specific immunocompetence that was activated after the exposure to the wild-type pathogen. The protective effect of the vaccine was evaluated considering that the immunized piglets were protected, with mild or absent clinical symptoms and pathological lesions observed and no bacteremia detected, when the challenges were conducted at days 20, postimmunization.

After the challenge at 20 dpi, all immunized piglets survived. In addiction, oral administration of the vaccine directly in the manger is less stressful for the animal than the use of needles and syringes.

4.7-Evaluation of intestinal Immunoglobulin-A titer in the immunization phase

Many studies have shown that the mucosal immune system, which is characterized by secretory IgA (S-IgA) antibodies, as the major humoral defense factor, contains specialized lymphoid tissues where antigens are encountered from the environment, are taken up and induce B and T-cell responses. This event is followed by an exodus of specific lymphocytes, which home to various effector sites such as the lamina propria regions and glands. These responses are regulated by T cells and cytokines and lead to plasma cell differentiation and subsequent production of S-IgA antibodies in external secretions. This knowledge has led to practical approaches for oral vaccine construction and delivery into mucosal inductive sites in an effort to elicit host protection at mucosal surfaces where the infection actually occurs. During the pre-challenge time, the piglets had been feeding with transgenic seeds of *Nicotiana tabacum*, expressing the antigenic proteins F18 and VT2e-B (6.6-7.4 µg and 34-37 µg per 10g seeds respectively).

The titer of fecal IgA-S was shown in Figure 23 and 24. From day 0 to 21, both experimental groups showed a progressive decrease of IgAs titer.

The luminal level of S-IgA (day 0 to 21) in piglets, probably related to maternal immunity, may decrease constantly due to the reduction of milk consumption.

The yield of IgAs had been measuring in their feces using specific ELISAassay as previously described. The results (Figure 23) showed a progressive decrease of IgA level in control group and an increase of intestinal IgAs in treated piglets from day 0 until 20th days.

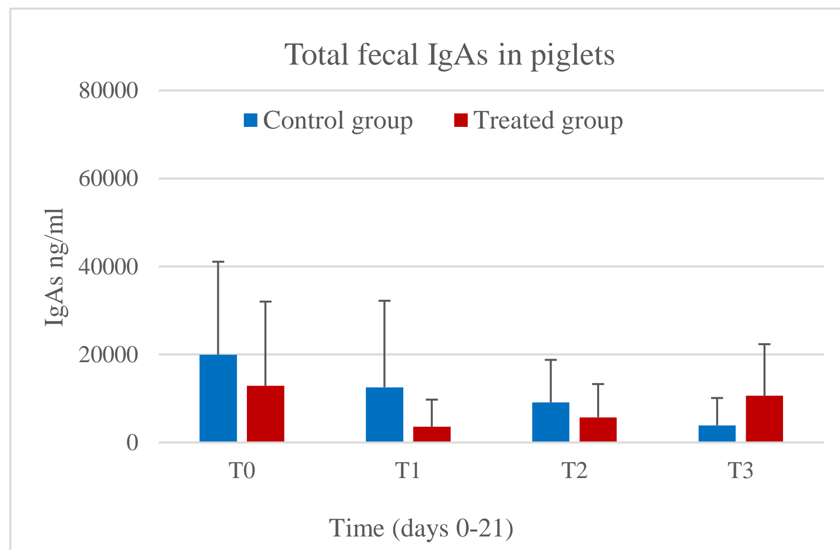


Figure 23: Evaluation of total mean intestinal IgAs titer of individual fecal samples collected from piglets in in pre-challenge period.

In Figure 24, the IgAs titer in piglets is shown. Each dot in the graph represent an individual immunoglobulin value during pre-challenge period. Secretory immunoglobulin A (S-IgA) is a protective molecule of the mucosal immune system. In the case of pigs, maternal S-IgA is supplied to piglets by the maternal colostrum and milk.

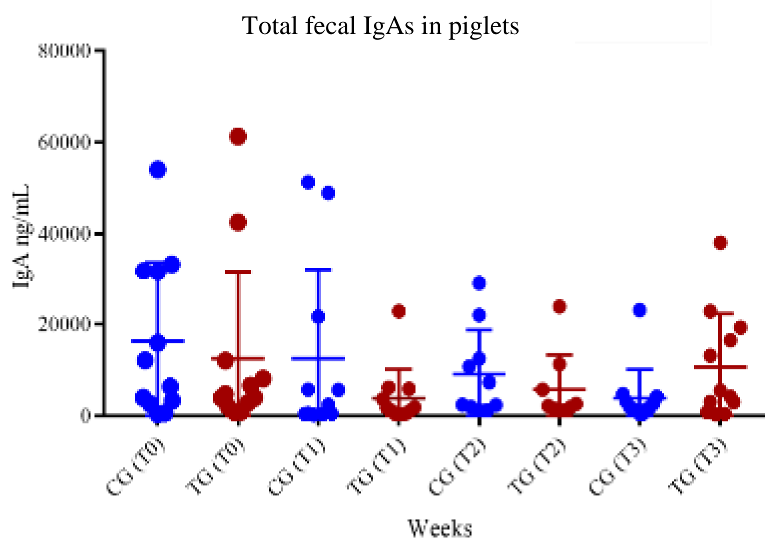


Figure 24: Evaluation of total intestinal IgAs titer of individual fecal samples collected from piglets in in pre-challenge period. CG: control group; TG: treated group.

However, regarding the treated group, which is different from other pigs in term of feed, from day 7th until 20th, the treatment group increased the fecal IgA level. Based on the ELISAassays treated group showed a higher fecal titer of IgAs

(+37%) at day 20th. These data might suggest improvement of the gut mucosal immune system and reflect the progressive increase in the local immune response actively involved in microbial infection. As observed in Rossi et al. (2011) the use of multicomponent vaccine, based on two important virulence factors of the VTEC strain, appeared to be more effective for the activation of the local immune response. However, fecal IgA titer did not reveal a statistical significance between experimental groups. The lack of significance of this finding is probably due to the large intra-individual variation in fecal immunoglobulin levels observed in our experiment, higher than that observed in preliminary studies performed on a mouse model (Rossi et al., 2003b). This aspect was influenced by the variability linked to the colostrum intake and by the fact that the piglets enrolled in this trial were not specially made for experimentation *in-vivo*. In addition, the number of enrolled animals was limited. The individual variation in local immune response is well-documented (Crawley et al., 2005). Fritz at al. (2012) also demonstrated the pivotal role of IgA+ plasma cells in the local immunity (specific and a specific) in a paper published in Nature.

4.7.1-Evaluation of anti-FedA IgAs and anti-VT2e-B IgAs in fecal samples

The *E. coli* F18 fimbriae allows adhesion to specific receptors on small intestinal villi and consequently the colonization can occur. The presence of the F18 receptor is genetically determined by an autosomal dominant gene and their absence makes pigs resistant to infection. Following colonization, VTEC strains produce the Shiga-like toxin type II variant (SLT-IIv), a vasotoxin that acts on vascular endothelial cells resulting in edema and subsequent neurological signs including ataxia, recumbence, and paddling movements, eventually leading to death. Colonization begins after adhesion of the bacteria with their fimbriae to the small intestine and stops when anti-fimbrial antibodies can be detected in the intestinal lumen of the infected pig. To protect weaned pigs, an oral vaccine is a suitable method to induce not only total S-IgA but also specific local immunity, since parenteral immunization does not induce protective immunity at mucosal surfaces (Bianchi et al., 1996).

Van den Broeck et al. (1999) reported that oral immunization of weaned pigs with purified F4 fimbriae protects them against subsequent $F4^{+}$ETEC infection. For $F18^{+}$ *E. coli*, however, only an infection has been reported to induce protective F18-specific antibodies at the small intestinal mucosa.

Treated group showed at day 20, after the immunization phase, a higher level of anti FedA subunit immunoglobulin-A, if compared to controls (Figure 25).

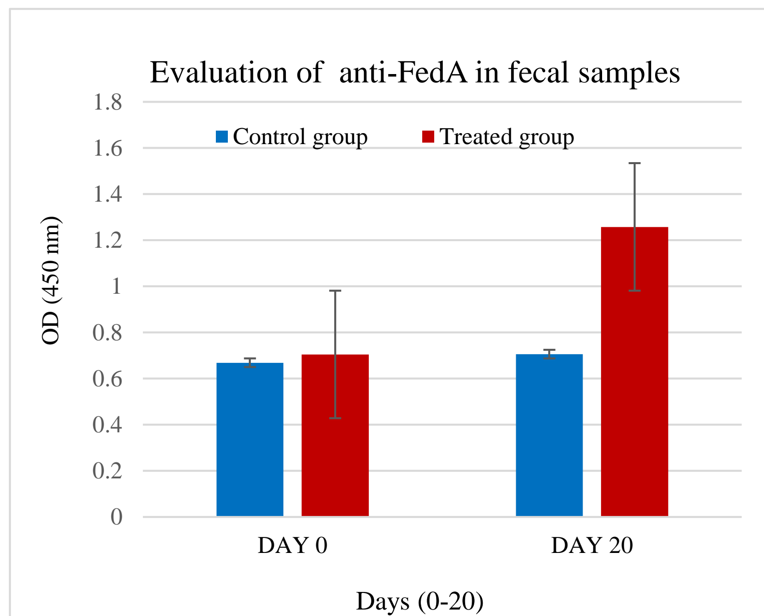


Figure 25: Evaluation of anti-FedA immunoglobulin A in fecal samples in the pre-challenge period.

Also anti-VT2e-B immunoglobulin-A level resulted at day 20 higher in the treated group. These data suggested a specific activation of the local immune system against VTEC strains (Figure 26).

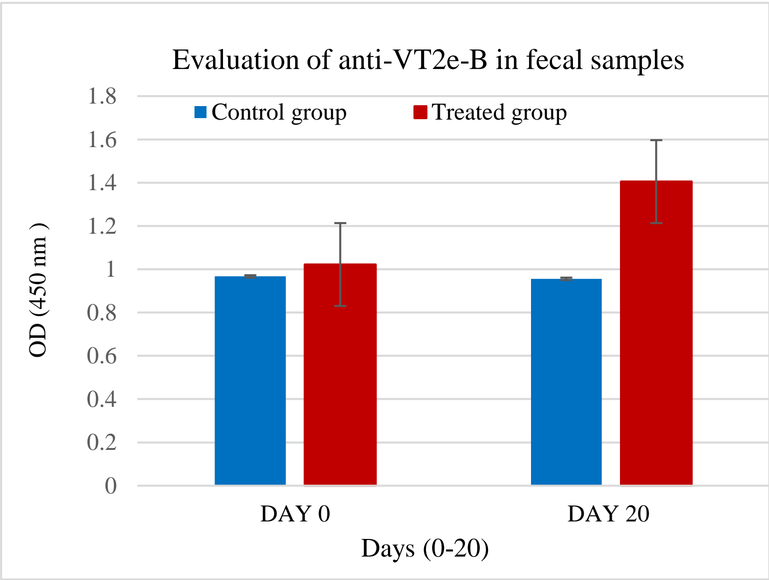


Figure 26: Evaluation of anti-VT2e-B immunoglobulin-A in fecal samples in the pre-challenge period.

5-CONCLUSIONS

Medical and Molecular Farming represent a new way to produce affordable contemporary drugs and make these globally available. The greatest target of the latter will be the prevention of infectious diseases, especially in underdeveloped countries where the access to drugs and vaccines has historically been inadequate. The idea that "prevent is better than care" gives new stimuli to develop innovative techniques in order to avoid outbreaks of infection diseases, the abuse of drugs, in particular antibiotics. As we move into the 21st Century and the demands for animal food products increase to meet the nutritional needs of a growing world population, alternative strategies to prevent and control animal diseases is a global issue and a critical component of efforts to promote global food security, and global health security.

This work was focused on the production of tobacco seeds as a model of edible vaccines against verocytotoxic *Escherichia coli* (VTEC) in piglets. Two lines of *Nicotiana tabacum* respectively engineered for the seed-specific expression of VT2e-B and F18 adhesive fimbriae, were previously obtained (Rossi et al., 2003a) as a model of edible vaccines against VTEC by agroinfection (*Agrobacterium tumefaciens* EHA105) using pBIpGLOB binary vector. *Nicotiana tobacco* is a model plant that makes it suitable as a model for edible vaccine. A first finding of the study was that engineered tobacco are able to produce an adequate amount of vaccinal seeds. The vaccinal seeds were seeded and cultivated in a Fitotron® growth chamber in optimal conditions. Analyses have been executed on the germination index of the plants and on the chemical composition of the seeds. It was evidenced that both the germination and the chemical composition have been proved to be congruent with the wild-type plant. However, considering the germination time, the engineered tobacco seeds have germinated four days after the wild-type plants. We hypothesized based on morphological analyses that this is probably because the engineered tobacco plants did not use inclusion bodies such as protein and lipid bodies. As expected, it could be that the random integration of gene into plant genome interrupted the

sequence of some genes involved in the metabolism or on the use of substances useful for the growth of the plant.

After the seed-collection period, it has been possible to obtain 450 gr of VT2e-B seeds and 470 gr of FedA seeds. The optimization of cultivation practices of tobacco plants had been studied for obtaining the maximal seed production. Anyhow, the obtained quantity of seeds results to be lower compared to a standard in-field collection, also the losses, related to the manual harvesting must be taken into account. Nevertheless, the obtained quantity was enough for further *in-vivo*-trials. Molecular analyses performed with the PCR technique that confirmed the presence of bacterial genes incorporated into genome of engineered plants. 20% of plants were screened, 100% of the enrolled samples resulted positive to the PCR. Further analyses with ELISA-assays confirmed the vaccinal antigens in seeds that make possible to perform the *in-vivo* trial. We then focused on strategy to administrate the seeds to the piglets. To guarantee a complete assumption of the treatment, the piglets have been fed with a mixture made of seeds and milk replaced powder directly placed in the trough to simulate a possible treatment in livestock. Since the tobacco presents an impermeable coat, the grounding became pivotal for the formulation of the experimental feed in order to assure a sufficient antigen exposure in the intestinal mucosa. The seed grounding has been performed at a low temperature to prevent protein degradation, which could occur at high temperatures (about 50°C). The efficacy of tobacco seeds-based oral vaccines directed against VTEC infection (authorization: 102/2015-PR) was evaluated through an *in-vivo* trial carried out in the Experimental Animal Research and Application Centre (pigs sector) in Lodi of the University of Milan. We found that piglets fed tobacco seeds expressing VTEC antigens have over all a better growth performances and clinical status after challenge with the O138 VTEC *E. coli* strain, compared to the controls. The oral delivery strategy appeared effective in reducing the development of clinical signs (respiratory, palpebral oedema, epiphora, vitality, fecal consistency, and rectal temperature) after challenge with O138 VTEC *E. coli* strain. We also found that total IgA and the specific IgA anti FedA and anti VT2e-B in piglets. At day 20 (the end of immunization period) in the piglets group that received vaccinal seeds the titre of total and specific IgAs were higher

than the control group that did not receive vaccinal seeds. Although the results were not statistically significant, (probably due to the small number of animals), the antigenic proteins administered to piglets in feed have stimulated an increase of production of IgAs in the gastrointestinal tract. Based on these results and considerations *Nicotiana tabacum* based vaccine could be a promising way to protect piglets against enteric disease caused by VTEC. The most important factor in moving plant-produced heterologous proteins from developmental research to commercial products is to ensure competitive production costs, and the best way to achieve this is to boost expression. Thus, considerable research effort has been made to increase the amount of recombinant protein produced in plants. This research includes molecular technologies to increase replication, to boost transcription, to direct transcription in tissues suited for protein accumulation, to stabilize transcripts, to optimize translation, to target proteins to subcellular locations optimal for their accumulation, and to engineer proteins to stabilize them. In addiction engeneered plants and vegetables can be used as delivery system to deliver pharmaceuticals and additives for gut health of humans and animals. Further studies are needed to better evaluate the correct dose of vaccinal seed. Dose-curve response studies should be performed, but this implies a production of an higher amount of engineered seeds. A further aspect to be considered is the mucosal immune response. The underlying mechanism of mucosal immunity has been deepened in an independent project in collaboration with the University of Denver (Colorado). The main goal of the project was to characterize the effects of different mucosal bacterial pathogens (two strains of *Haemophilus influenzae, Moraxella catarrhalis* and *Staphylococcus aureus*) on the isotype of antibody production from human B cells. Further applications of this technology in the future would make it possible to produce modern drugs in plants. Plants are easy to grow; they produce a large amount of biomass, and are appropriate for a cheap production of high quality and bioactive recombinant proteins. Besides, the mechanism of N-glycosylation in plants and mammals is similar until the developing *N*-glycan reaches the Golgi apparatus. One of the main features of molecular pharming that distinguishes it from other industrial concepts - and has debatably worked against its profitable development - is that there isn't a standardized platform or a single technological foundation. Instead, different platforms and technologies have been developed

with diverse and sometimes overlapping benefits, connected only by their employment of plant tissues (Stoger et al., 2014). The platforms vary from plant cells or simple plants growing in bioreactors containing fully defined synthetic media, to whole plants growing in soil or in hydroponic environments. These technologies encompass a stable transgene integration and transient expression when using bacterial, viral, or hybrid vectors (Fischer et al., 2013). An important discovery was achieved in 2012, when the first molecular pharming product was approved for humans' use. In particular, this was the enzyme taliglucerase alfa, a recombinant form of the human glucocerebrosidase - developed by Protalix Biotherapeutics – used to treat the lysosomal storage disorder known as Gaucher's disease. Two clinical trial applications for plant-derived pharmaceuticals were also approved in the European Union (EU), one for insulin production in safflower (developed by SemBioSys Genetics) and another for an HIV-neutralizing monoclonal antibody production in tobacco, significant because of its public funded consortium (Pharma-Planta) development. In either case, new manufacturing procedures based on engineered plants were developed to guarantee the compliance with pharmaceutical good manufacturing practices (GMP). Moreover, Mapp Biopharmaceutical Inc., a company situated in San Diego, CA, USA produced a drug in tobacco leaves called ZMapp, which has been used to fight the 2014 Ebola virus outbreak in Africa (Arzten et al., 2015). The main influence of molecular farming is therefore likely to be the delivery of new strategies for medical involvement that removes some of the limitations of conventional manufacturing processes, instead of the indiscriminate replacement of current manufacturing infrastructures.

6-SUPPLEMENTARY STUDY:

In order to examine in depth, the aspects related to local immunity, an independent project to characterize the effects of different mucosal bacterial pathogens (two strains of *H. influenzae, M. catarrhalis and S. aureus)* on the isotype of antibody production from human B cells has been developed. The potential "decoy" stimulation by surface bacterial proteins that is proposed to stimulate B cells non-specifically by cross-linking surface antibody by the constant Fc region rather than binding these bacteria by the antigen-specific Fab variable region. This part of the study has been conducted in collaboration with the Department of infectious diseases, University of Denver, Colorado.

6.1-ISOTYPES DISTRIBUTION OF ANTIBODIES IN RESPONSES TO T CELL INDEPENDENT STIMULATION

6.1.1-Mucosal microbiota

Indigenous microorganisms colonize in children and adult human the skin and the mucosal gastrointestinal canal (GI) (Hentges, 1993; Tannock, 1995*).* The normal skin, the mucosal surface, the lumen of the alimentary canal and the gastrointestinal tract; harbor microbial cells in numbers that in aggregate can be estimated over 10^{14}. The population of various bacterial species in the biota differ in adults from those of children and middle-aged adults. In addition, the interaction of bacterial species with the immunologic system in children and in adults may be different. These different interacting ways may complicate the understanding of how the mucosal microbiota interacts with the mucosal systems.

In particular, in the nasopharynx and oropharynx tract the indigenous biota of the anterior nares of the normal adult generally reflect the bacteria biota in the skin. With normal procedures, predominating species in the skin biota are the gram-positive, aerobic genera *Staphylococcus* and *Corynebacterium*, and

anaerobic, gram-positive genus *Propionebacterium*. Gram-negative genuses such as *Haemophilus and Moraxella* are reported to be numbers of the indigenous nasopharyngeal and oropharyngeal biota (Table 34).

Gram characteristic	Genus	Location[b]
Not applicable	*Mycoplasma*	OP
Positive	*Staphylococcus* *Corynebacterium* *Propionibacterium*[c]	AN AN AN
Negative	*Moraxella* *Haemophilus* *Neisseria*	NP OR/NP OR/NP
[b] OP, oropharynx; AN, anterior nares; NP, nasopharynx [c] Strict anaerobe. From Tannock et al., (1995) and Hentges et al., (1993)		

Table 34: Nasopharyngeal and oropharyngeal biota.

The mucosal microbiota in various areas of the body has been known for many years to have the capacity either to limit the growth of, or to kill, certain transient microbial pathogens entering their habitats. Some strains of *S aureus* in the nasopharynx can prevent pathogenic strains of the same bacterium from colonizing the mucosal surface (Tannock, 1995). Moreover, for several years researchers have been focused in different studies on the mechanism by which the indigenous biota on mucosal surface interacts with the body's immunologic system. In particular, two major aspects have been considered the immunologic system is tolerant of the indigenous microbiota, the microbiota communicates with the cytokines network. Many researchers were involved on antibody responses to antigens of bacteria in mucosal surfaces. In 1983, Berg discussed “natural antibodies and concluded that the serum and secretions of healthy adult human subjects contain in low titers IgAs, IgGs, IgMs, IgDs antibodies reactive with antigens of indigenous bacteria. In particular, he compared antibody responses to indigenous and nonindigenous bacteria either into or given orally to GF rodents. He concluded that the results supported a hypothesis that certain bacteria indigenous to the mucosal tracts of body do not elicit immunologic responses as well as do various nonindigenous bacteria. Another main aspect in the relationship between bacteria and mucosal immunologic system is that the indigenous microbiota communicates with the cytokines network. In a famous review Henderson et al. (1996), assessed the ability of multicellular complex organisms to harmoniously live with body's mucosal microbiota. It is based on

mutual signaling involving eukaryotic cytokines and prokaryotic cytokines like-molecules. These bacterial products are being listed such as proteins toxin cell surfaces polysaccharides, endotoxin associated proteins, lipoproteins, peptidoglycans and many others.

6.1.2-Lymphoid organs of the respiratory tract

The upper and the lower respiratory tract airways are continually exposed to antigen challenges. This is reflected in the specialized cells and lymphoid organs in the nasal and buccal mucosae and in the airways until to lungs. In the human nasopharynx tract, a set of lymphoid tissue surrounds the nasal. In contrast to this highly organized and strategically positioned oro-nasal complex of lymphoid tissue, in lung of human only poorly developed lymphoid tissue can be found and oral passages in the pharynx. This specialized part upper respiratory tract is generally called Waldeyer's ring and includes the adenoids and the tonsils (Pherry and White, 1998).

6.1.2.a-Structure of NALT (Nasal–associated lymphoid tissue)

The organization and structure of NALT have been studied most extensively in small rodents such as rats and mice. It is situated in the floor of nasal cavity as a pared organ, slightly bulging out into the nasal cavity right at the entrance of the pharyngeal duct. At those placed where the respiratory epithelium covers the NALT it is characterized by the presence of specialized microfold M (Karchev and Kabakchiev, 1984). These M cells are found in all mucosal epithelia covering lymphoid aggregate, such a tonsil, Peyer’s patch, and BALT. In addition to M cells, which can be found in small clusters or alone, the epithelium covering the NALT contains a few mucous goblet cells. Whereas most of the epithelial cells lining the nasal cavity are determined to keep antigens and microorganism out, being protected by a mucus layer and constant activity of their microvilli, the M cells have evolved special mechanisms to transport macromolecules and particles across the epithelium. The M cells seem to be specialized in transcytosis (Kraehenbuhl and Neutra, 2000). Bound particles such as bacteria and viruses induce changes in the apical membranes and cytoskeleton, after which phagocytosis take s place. Reorganization of the cells membrane and extension of the cellular process around the particles have

been described, and the particles are transported through the cells in phagocytic vesicles (Owen et al., 1999). Smaller particles and macromolecules are taken up through clathrin-coated pits or by receptor mediated phagocytosis. Nevertheless, there seems to be an active interaction between the M cells and underlying lymphocytes because after depletion of lymphocytes by irradiation the intraepithelial pocket of the M cells in Payer's patches is lost (Ermak et al., 1989) (Figure 27). Data on the phenotype of the lymphocytes that reside in the pockets of the M cells in NALT are limited, but in Payer's patches the majority of cells that are localized in the M cells pockets are $CD4^{+}$T cells. B cells are also found in the pockets. The number of B cells in NALT roughly equaled the number of T cells.

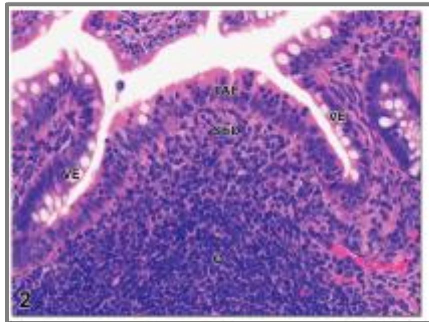


Figure 27: Peyer's Patch, small intestine Sprague–Dawley rat, male, 31 days old. The follicle-associated epithelium (FAE) contains clusters of lymphocytes, which may be associated with an M-cell. It is also slightly attenuated and has fewer goblet cells relative to the villous epithelium (VE). (Toxicologic Pathology, 34:599–608, 2006 Copyright C. Mark Cesta, 2006)

6.1.3-Function of the NALT

In the past years, evidence has been accumulating that NALT is an important inductive site of immune responses after nasal immunization. Indeed, immunization via nose is an effective way to induce mucosal immune response s at remote effector site, and a role for NALT in the induction of these responses has been recognized. A scheme with and position of NALT in relationship with nose-draining lymph nodes and the nasal epithelium is shown in Figure 28 and is based on the work of Kupfer et al. (1992). Particulate antigen is predominantly taken up by M calls of the follicle-associated epithelium of the NALT. Whereas soluble smaller antigens are taken, up by the nasal epithelium and drain into lymph nodes directly or associated with DC (dendritic cells). In the lymphoid organs, B cells will preferentially switch to IgA plasma-blasts, after which they leave the lymphoid tissue to localize as effector plasma cells in and underneath the epithelium of the nasal passage

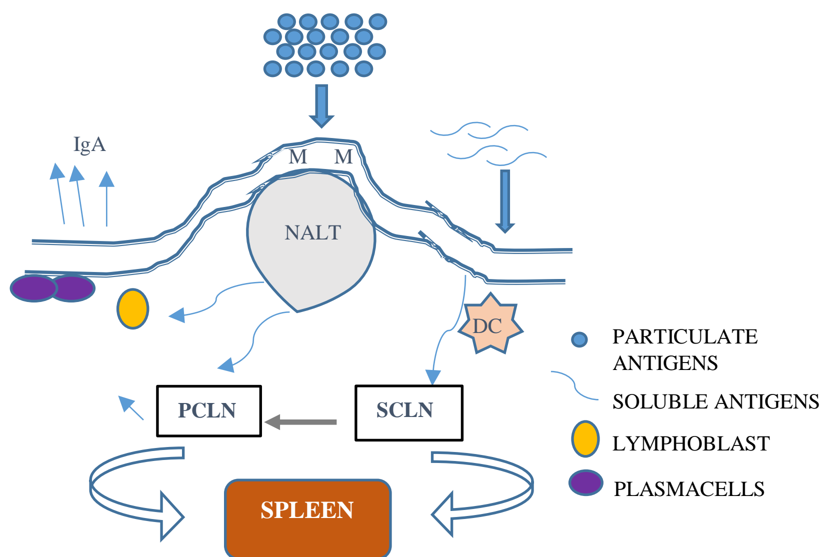


Figure 28. Scheme showing the interaction between NALT, nose drawing lymph nodes and the spleen PCLN, posterior cervical lymph node. SCLN, superficial cervical lymph node.

6.1.4-The B Cell Development

6.1.4.a-B cell development and immunoglobulin class switching

The bone marrow, in both mouse and human being, is the primary site of early B cell maturation. At this location, B cells pass through a series of antigen independent maturational stages defined by the presence or absence of certain surface and cytoplasmic markers and by specific stages of Ig variable region heavy and light chain gene rearrangements and expressions. In each B cell, the selection and assembly of the genes encoding the variable region of the Ig molecule occur as a result of a random recombination process in which the selected variable region (V) diversity(V) and Joining (J) gene segments come together to form a single variable region gene complex. The heavy chain V region gene is formed by two rearrangements. First the joining of D and J genes occur, followed by the joining of a V gene to the fused DJ sequence whereas the light chain variable region gene is formed by a single rearrangement joining V and J genes. This variable region recombination ultimately results in the production of specific heavy and light chain proteins that can then be assembled to produce the unique Ig molecules that characterize each B cell and its progeny. The V(D)J recombination is tightly controlled by the transcriptional regulation of the recombinase genes that encode RAG-1 and RAG-2 proteins. The most immature B cells contain unarranged heavy and light chain variable region genes. Upon recombination of the heavy chain variable region genes, μ heavy chain encoded by the rearranged variable genes linked to the downstream Cm gene are present in the cytoplasm and on the cell surface in combination with surrogate light chain. Then following completion of the light chain gene rearrangement, both heavy and light chains are produced, and a complete IgM or IgD molecules can be assembled from the organized variable region genes linked to Cμ or Cδ heavy chain genes The immature IgM B cells that co-expresses high levels of surface IgM and low levels of surface IgD can then be formed. These immature B cells leave the bone marrow and pass via the circulation to various peripheral lymphoid tissues, including the spleen, Peyer's patches (PPs) of the intestine, and lymph nodes. Upon specific immune challenge, a humoral immune response can be induced that is characterized by

the formation of activated B cells. Once antigen bind the specific antigen receptor on the surface of B cells, the clones committed to producing antigen-specific Ig are selectively expanded After that SIgM positive B cells might undergo terminal differentiation to become IgM-secreting plasma cells or class-switch recombination (CSR) into B cells producing another Ig isotype. Somatic hyper-mutation (SHM) might also take part at this stage of B-cell development, resulting in intraclonal diversity and enhanced antibody affinity or specificity. The encounter of a particular antigenic array serves to enhance the host's state of preparation of its immune response to successive encounters with the same antigenic stimuli. This is practiced by providing a memory B cells response characterized by B cells that have antigen receptors with enhanced affinity for antigen and that produced classes of Ig with more specialized effector functions through a mechanism called Ig class switching.

Immunoglobulin class switch: molecular events. During the early stage of both B cells and T cells differentiation, V(D)J recombination allows the assembly of variable (V), diversity (D), and joining(J) segments of the V exon of the immunoglobulin and T cells receptor genes, giving rise to diverse repertoires of B and t cells, each expressing receptors for specific antigen. Mature B cells that have finished functional V(D)J recombination of both heavy (H) and light (L) chain genes and expressing IgM on the surface migrate to the secondary lymphoid organs, where they allowed to encounter antigens. Activate B cells proliferate and differentiate into plasma cells or memory B-cells. During this peripheral differentiation B cells undergo a second wave of remodeling the immunoglobulin loci with CSR and SHM as mentioned before. These unique events are responsible for isotype switching and affinity maturation. These two recombination events are regulated by a single molecule, activation induced cytidine deaminase (AID) (Muramatsu et al., 2000) (Figure 29).

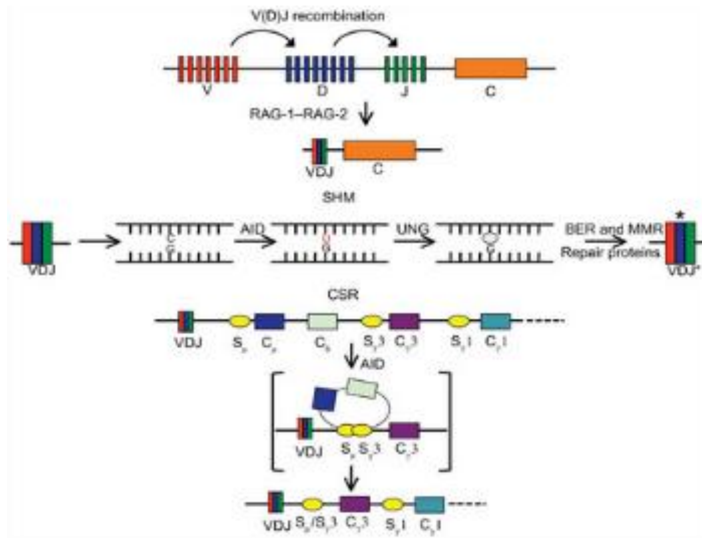


Figure 29: Antibodies diversification. (**a**) A deletional recombination event between individual V, D and J segments creates the variable region of the immunoglobulin gene. This process is catalyzed by the RAG-1–RAG-2 recombinase complex and occurs in an antigen-independent way. C, constant. (**b**) SHM, the first of two secondary antibody-diversification processes, results in the accumulation of point mutations in the recombined variable region. AID initiates this process through the deamination of cytidine to uridine, followed by removal of the uracil base by UNG and repair by several base-excision repair (BER) and mismatch-repair (MMR) enzymes. The asterisk indicates the rearranged, mutated variable region. (**c**) CSR completes the secondary antibody diversification and results in the exchange of the default constant region, C μ (IgM), for one of many downstream regions (C γ3 (IgG3) is presented here. AID is thought to initiate this process through deamination of bases in the switch (S) region (yellow circles) upstream of each constant region, resulting in the formation of DSBs and recombination (Muramatsu et al., 2000).

6.1.5-Outline of molecular mechanisms for CSR

The immunoglobulin C_h locus consists, of an ordered array of C_H genes, which, except for Cd, are flanked at their 5' region by unique repetitive sequences called S regions (Honyo and Kataoka, 1978; Kataoka et al., 1980; Davis et al., 1980; Rabbitts et al., 1980). The presence of the S region is required for CSR. Indeed, artificial switch substrates lacking S sequences are unable to perform class switch recombination. (Leung and Maizels, 1992). CSR take place between S regions (region specific S-S recombination). It requires double strand breakages, one in S and other in one of the downstream S regions, and it results in looped –out deletions of the intervening DNA segments (Iwasata et al., 1990). It is followed by repair and ligation of the broken DNA ends and by activation of the ubiquitously expressed nonhomoulogous end –joining (NHEJ)

repair system (Rolik et al., 1996; Casellas et al., 1998; Manis et al., 1998) the looped out circular DNA contains an I promoter that is still responsive to cytokines and directs production of I-Cμ transcripts called circle transcripts (CTs). These CTs serve as hallmarks for active CSR *in-vitro* (Fagarasan et al., 2001). The Cμ gene is located at the at the V_H proximal end of the C_H gene cluster, so that CSR between Sμ and another S region located 5' a C_H gene brings that particular C_H gene adjacent to the V_H exon. The result is a "switch" of the immunoglobulin isotype from IgM/IgD to IgG, IgA, or IgE having the same antigenic specificity but with different biological properties. The molecular process that underly switch differentiation is not random event but is induced by external environmental factors (McIntyre and Strober, 1999). Initiation of CSR requires B cell activation by antigens and cytokines secreted by a variety of cells, such as activated B cells and the cytokines milieu to which the B cells are exposed. Some antigens and some anatomic locations promote isotype-restricted responses, as shown by the preference for IgA development in GALT (Cebra et al., 1998). Signal transduction through surface IgM, Toll-like receptor, CD40, other TNF family member receptor (BCMA), and cytokines receptor on B cell induces two essential events that are required for CSR selection of a target region S and induction of AID (Zalazoswki et al., 1995)

6.1.5.a-Role of AID in class switch recombination

As discussed previously, the selection of S Target S region is mediated by transcription from the particular I promoter of mediated by transcription from the particular I promoter of that S region, and this event is an absolute requirement for CSR. Cytokines stimulation induces not only germline transcription associated with chromatin opening but also activation of new genes involved in CSR such as AID (Kinoshita et al., 1998). *In-vivo,* AID is strongly expressed in B cells located in the germinal center, the anatomic site where CSR and SHM take place with high efficiency. The essential role of AID on CSR was revealed by loss-of-function studies in both mice and humans. Mice AID deficient cannot produce any Ig Isotype other IgM, which is abundantly secreted under both immunized and non-immunized conditions. CSR is not detected in AID-deficiency B cells even after stimulation *in vivo*.

Neverthless these B cells proliferate normally and express normal germline transcripts of all downstream region in response to cytokines stimulation. Human AID deficiency, called hyper-IgM syndrome type II, shows an identical phenotype to AID deficiency in mouse, clearly indicating that AID is required for both CSR and SHM (Durandy and Honijo, 2001).

6.1.6-The Human Mucosal B-cell System

Lymphoid cells are located in three histologically distinct tissue compartments at the mucosal surface; the immune-inductive organized mucosa-associated lymphoid tissue (MALT), the lamina propria or glandular stroma, and the surface epithelia and Payer's patches in the distal small intestine. Typical MALT structures are believed to be a main source of conventional B2 sIgA-expressing primed mucosal B cells. Primary lymphoid follicles contain recirculating naïve B lymphocytes (sIgD$^+$ IgM$^+$), which pass into the network formed by antigen–capturing follicular dendritic cells (FDCs). Primary follicles are turned into secondary follicles by the germinal center reaction. In humans, this process has been extensively studied in tonsils by MacLennan, (1994) and Liu and Arpin, (1997), but much relevant mechanistic information relies on observation of lymph nodes and the spleen from immunized animals. Germinal centers are of vital importance for T cell-dependent generation of conventional (B-2) memory B cells, affinity maturation of the B cell receptor (BCR), and Ig class switching. It has been show that naïve B cells are first stimulated at the edge of the primary follicle by cognate interaction with activated CD4 T cells, which have previously been presented with processed antigens by MHC class II expressing DCs. The B cells then re- enter the follicles to become proliferating sIgD$^+$ IgM$^+$ CD38 germinal center "founder cells" as noted in human tonsils. Such initial stimulated B cells produce mutated IgM and some IgG antibodies of low affinity that can bind circulating antigen; the resulting soluble immune complex subsequently becomes deposited in the FDCs, where the antigen is retained for prolonged periods to maintain B-cell memory. Such a role for IgM in the induction of secondary immune responses with antibody affinity maturation has been strongly supported by observation in knock-out mice lacking natural (non-specific) background IgM antibodies. The complement receptor CR1/CR2 is considered a cell surface molecules that plays

a crucial role in the germinal center reaction. CD21 is expressed abundantly on both FDCs and B cells and may function by localizing antigen to the FDC network and/or by lowering the threshold of B-cell activation via recruitment of CD19 into the BCR. The germinal center can be divided into different compartment in which antigen –dependent selection of B cells takes place. Stimulation in the dark zone produces exponential growth of B cell blasts positive for Ki-67 nuclear proliferation marker. (Brandtzaeg and Halstensen, 1992) The resulting centroblasts somatically hypermutate their Ig-variable (V) region genes and give rise to sIgD-IgM^{+}CD38$^{+.}$

Some centrocytes with specificity for exogenous antigens undergo apoptosis unless selected by high affinity binding to FDCs via their sIgM/BCR. The importance of cognate interaction between B and T cells is well documented by the fact that no germinal center is formed when CD40-CD40L (CD54) ligation is experimentally blocked (Lindhout et al., 1997). Moreover, this ligation promotes switching of the Ig heavy-chain constant region (C_H) genes from Cμ to downstream isotypes, while apparently representing a negative signal for terminal B cells differentiation within the follicles. Interaction between CD27 on CD38^{+} germinal center B cells and CD27L in T cells may be a determining event (Jung et al., 2000).

Exit of B cells from germinal center.

Migration of activated B cells from the germinal center is most likely directed by chemokynes, and the actual cues may be extrafollicular ligands for CCR7. Thus, activated germinal center B cells downregulate CXCR5 and upregulate CCR7. Most MALT-induced sIgD^{-} IgM^{+} CD38^{-} putative memory B cells migrate continuously out of the germinal center to sites such as the tonsillar crypt epithelium or to Payer's patches M cell pockets. (Yamanaka et al., 2001)

The switching process.

Following activation, naïve B cells usually first change their BCR composition from IgD^{+} IgM^{+} to become $sIgD^{-}$ $SIgM^{+}$ memory cells and may then switch to another class such as IgGs or IgAs. During plasma cell differentiation the BCR is gradually lost, together with several other B-cells markers, particularly the CD20 and then the CD19. Activation induced cytidine deaminases (AID) play an essential role in the process where B cell differentiation downstream (5' 3') switching of C_H genes on chromosome 14, from IgM ($C\mu$) to other isotypes (Flanagan and Rabbits, 1982). The tonsillar germinal center reaction involves extensive switching and some terminal differentiation of effector B cell (Dahlenborg et al., 1997). Thus, immunoistochemical studies have revealed a substantial although variable number of intrafollicular Ig producing plasmablasts. Predominantly witch cytoplasmic IgG (55% to 72%) or IgA 13% to 18%. Both these germinal center immunocyte classes, and also those producing IgM and IgD, are often associated with detectable cytoplasmic J-chain expression (IgG 36%, IgA 29%, IgM, 55%and IgG, 82%), in normal palatine tonsils as well as adenoids from children and adult.

Regional isotype-switch mechanisms differ.

The cytokines profiles and other micro environmental factors that determine isotypes differentiation of B cells are likewise obscure. The fact that IgA1 subclass dominates IgA responses both in tonsils and in regionally related exocrine tissues supports the notion that mucosal B cell differentiation in this body region mainly takes place from $SIgD^{-}$ $IgM^{+}CD38^{+}$ centrocytes by sequential downstream C_H gene switching. Conversely, the relatively enhanced IgA2 expression in Payer's patches and the distal gut altogether including the mesenteric lymph nodes (Kett et al., 1986), could reflect direct switching from $C\mu$ τo $C\alpha 2$ with excision of intervening C_H gene segments. The germinal center reaction generates relatively more intrafollicular J chain-positive IgA cells in human Payer's patches and in appendix than in tonsils (Brandtzaeg et al., 1999b). Also in juxtaposed extra follicular GALT compartments, IgA immunocytes are equal to exceed in numbers their IgG counterparts, whereas in tonsils there is a more that two-fold dominance of IgG immunocytes outside of the follicles (Brandtzaeg, 1987). Therefore, the drive for switching to IgA

and expression of J chain is much more pronounced in GALT than in tonsils. Perhaps GALT is distinct from other MALT structures because of its special accessory cells or a particular cytokines profile.

6.1.6.a-Microbial impact on mucosal B cell

A regionalized microbial impact on mucosal B cell differentiation may be exemplified by the unique S $IgD^{+}IgM^{-}CD38^{+}$ subset identified in the dark zone of palatine tonsillar germinal center (Liu and Arpin, 1997). This centroblast showed a deletion of the Cμ and Sμ gene segments therefore giving selectively rise to IgD immunocytes by non-classical switching. In the literature, there is molecular evidence of preferential occurrences of B cells with Cμ deletion also in normal adenoids and secretory and secretory effector tissues of the upper aero digestive tract but virtually never in the small intestinal mucosa (Brandtzaeg, 2002). Most strains of *Haemophilus influenzae* and *Moraxella catarrhalis, which* are frequent colonizers of the nasopharynx, produce an IgD binding factor (Protein D) that can crosslink SIgD/BCR (Ruan et al., 1990). In this manner it is possible that $sIgD^{+}$ tonsillar centrocytes are stimulated to proliferate and differentiate polyclonally, hereby driving IgV^{-} gene hypermutation and Cm deletion (Liu et al., 1991). Such regional microbial influence on B-cell differentiation is supported by observation that Cμ deletion is more frequently detected in diseased than in clinically normal tonsils and adenoids (Branstzaeg et al., 2002) and there is an increased number of extra-follicular IgD immunocytes in recurrent tonsillitis and adenoids hyperplasia (Brandtzaeg, 1987).

6.1.7-Bacterial interaction with mucosal epithelial cells.

6.1.7.a-Bacterial interaction with mucosal epithelial cells in the respiratory tract

The human respiratory tract (RT) can be divided in two regions: nasopharynx and lung, which are sterile. Epidemiological studies have demonstrated that while many bacteria are capable of infecting the lower RT only few species do so commonly (Mandell, 1995). These pathogens cause two distinct types of diseases. Infection limited to the airways is exemplified due to *Bordetella*

pertussis that cause whooping cough (Cooter et al., 2001). Infection of the alveoli produces pneumonia. In most development countries, the most common agents of community-acquired pneumonia are *Streptococcus pneumonia*e, *Haemophilus influenza, Moraxella catarrhalis* and *Mycoplasma pneumoniae.*

The respiratory pathogens initially colonize the nasopharynx and subsequently are aspirated into lungs. All healthy children and adults are colonized repetitively and asymptomatically. Colonization progress to disease commonly in individuals who are immunocompromised underlying conditions (*e.g.* cystic fibrosis, asthma, chronic pulmonary disease), or have habits or lifestyles that impair the normal functioning of the innate defense of the lung (*e.g.* smoking or alcoholism) (Mandell, 1995). As those in the gastrointestinal tract, most bacteria in the nasopharynx are commensal, land exist in balance with the host. The presence of these bacteria is also most likely beneficial, if simply to occupy a niche and prevent colonization by more pathogenic strains. The signature characteristic of bacteria that causes lower RT infection is the ability to adhere to and invade mucosal epithelial cellsn(MECs). Either ciliate cells of the airways or type I and II alveolar cells in the terminal air sacs. To gain access to MECs, pathogens must overcome the gauntlet of innate and specific defenses present in mucosal secretion. These defenses routinely protect the host from the onslaught of commensal and pathogenic bacteria introduced into RT daily.

Cilia and the mucin layer. Mucus is the primary innate defense protecting mucosal epithelial cells from microorganisms and serves as a physical barrier to infection (Knowel et al., 2002). Beating of cilia of epithelial cells transports mucus and entrapped particles to the mouth, where they can be swallowed and killed in the stomach. Mucin is a complex mixture of very diverse, high molecular weight, glycosylated macromolecules that, by virtue of carbohydrate diversity, is capable of binding almost any particle that lands on the airway surface (Klein et al., 2000). Bacteria evade mucin entrapment by reducing the number of receptors in the surface or by enzymatic digestion of mucin by glycohydrolases. Digestion of mucin reduces the viscosity of the mucus, enabling bacteria to penetrate to the epithelial surface. These enzymes also serve to cleave terminal carbohydrate structures from the surfaces of MECs, exposing previously cryptic antigens that then function of receptors for the

bacteria (Tong et al., 2000). The most direct mechanism for protecting the host from bacteria is to kill prior to their contact with MECs. Accordingly, the mucus layer, in addition to being a physical barrier, is a chemical shield exposing bacteria to an extensive array of antimicrobial peptides and inhibitory proteins. Antimicrobial factors present in mucus, include complement, cathepsin G, defensins, surfactant, lysozyme, and lactoferrin (Brogden, 2000).

Defensins are secreted by epithelial cells and damage the bacteria cell membrane causing blebs or lytic pores (Leherer et al., 1991). Bacterial strategies against defensins focus at either neutralizing the peptides or evading those (Brogden et al., 2000). Strategies, including altering receptors for defensins (such as outer membrane proteins, LPS, teichoic acid), adsorbing charged molecules the repel the peptides, or actively transporting the defensins away from the bacterial surface. Another important factor in the RT secretions is complement. LPS ion gram-negative bacteria activate the complement. LPS on the gram-negative bacteria activate the alternative pathway by binding C3b and C5b, which then serve a nucleus for the formation of the membrane attack complex (MAC) (Downey et al., 1997). Alternatively, other components such as IgG and C-reactive protein (CRP) once bound activate the classical complement pathway. Bound complement opsonized bacteria for phagocytosis, while soluble C3a and C5a serve potent chemotactic molecules for neutrophils and macrophages (Eredei et al., 1997). Some gram-negative bacteria circumvent complement by attaching sialic acid to their surface. Sialic acid serum protein H, which in turn binds protein I. Protein I degrades C3b, thus preventing the formation of the C5 convertase (Rautemaa et al., 1999). This sialic acid based defense is a common strategy with many variations. *Naisseria gonorrheae* displays sialic acid as O-antigen, while N*aisseria meningiti*s display sialic acid as a major capsular component. In addition, some bacteria produce an elaborate array of virulence factor, including a protease, lipase, hemolysin, and cytotoxin. A critical element making the mucous trap for bacteria effective is the expulsion of secretions from the lower RT by the ciliary escalator. Thus, it is not surprising that pathogens secrete toxins that can stop ciliary beating. The most specific of these toxins, tracheal cytotoxin, is derived from the cell wall of B pertussis and is exquisely potent and specific at killing

ciliated cells (Wilson et al., 1991) to bacterial products is dependent on a family of membrane proteins termed.

TOLL-like receptors (Medzhitov et al., 1997). TLRs activate the signaling pathway that initiates the innate immune responses to infection and begin the process to adaptive immunity. These receptors play a major role in phagocytosis cells such as alveolar macrophages and neutrophils but also dictate responsiveness of lung epithelial cells (Wang et al., 2002). TLR responses are tailored for the class for the class of the infectious agent, (Kopp et al., 1999). For example, TLR4 recognize LPS and as such is activated by gram-and negative bacteria. TLR2 recognized components of gram-positive bacteria such as peptidoglycan and teichoic acid. TLR9 recognized nucleic acid motifs. Activation if TLRs triggers intracellular signals, leading to induction of the innate response (defensins, cytokines , the expression of PAF and ICAM) and recruitment of neutrophils and effector cells to clear the infection. During the invasion process, several mucosal pathogens trigger apoptosis.

6.1.8-Briefly overview of microorganism of the respiratory tract.

Haemophilus influenzae **(Hib)**

Hib is a respiratory tract bacterium and is considered to be commensal particularly in preschool children. This type of bacteria is divided into encapsulated strains (serotypes a–f) and unencapsulated (NTHi*),* that is, nontypeable *H. influenzae* (NTHi). An efficient vaccine against *H. influenza* type b (Hib) was introduced in the 1990s, and ever since the incidence of Hib infections has decreased dramatically. NTHi is after pneumococci one of the leading causes of acute otitis media in children (Murphy et al., 2009). In adults, NTHi causes sinusitis as well as bronchitis, and is frequently found in patients with chronic obstructive pulmonary disease, both during stable disease and acute exacerbations. Although *H. influenza* is associated with an array of respiratory tract infections, NTHi is also considered as a nasopharyngeal commensal of mainly preschool children (Faden et al., 1995). Immune evasion by *H. Influenza* includes the production of immunoglobulin A (IgA) protease and circumvention of complement-mediated attacks by accumulation of

complement inhibitors and delayed deposition of complement proteins (Vitovski et al., 2002) Another interesting mechanism, which often is implicated as a binding to immunoglobulins. This phenomenon implicates binding to the conserved regions of immunoglobulins different from conventional antigen binding sites. Immunoglobulin D (IgD) binding has been demonstrated for Hib (Samuelsson et al., 2007).

***Moraxella catarrhalis* (Mcat)**

Mcat is a Gram-negative human specific pathogen of the respiratory tract. (Figure 30). For a long time, the potential pathogenicity of *M. catarrhalis* was ignored and the species was mainly recognized as a commensal. However, *M. catarrhalis* is now acknowledged as a causative agent of respiratory tract infections in children and adults leading to a considerable morbidity. *M. catarrhalis* is the third most important pathogen causing otitis media in children and is responsible for up to 20% of cases with a majority of the children experiencing at least one episode of otitis media by the age of 3 years (Verduin et al., 2002). Other respiratory tract infections in children associated with *M. catarrhalis* include sinusitis, conjunctivitis, bronchitis and pneumonia (Ahmed et al., 2008). The carriage rate of *M. catarrhalis* is low in healthy adults and if infected, adults are usually predisposed to lower respiratory tract infections with *M. catarrhalis* in case of compromised situations such as chronic obstructive pulmonary disease (COPD) and previous episodes of pneumonia. Colonization of the nasopharynx by *M. catarrhalis* and other predominant respiratory tract pathogens such as non-typeable (NTHi) and *Streptococcus pneumoniae* begins at a very early age in the life and at least 50 % of children are colonized by the age of 2 years. Colonization not only provides the potential pathogens an entry point to various associated tissues including the ear, sinuses, larynx and lower respiratory tract but is also means of spreading in the community via nasal secretions.

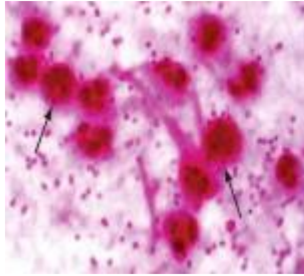


Figure 30. *Moraxella catarrhalis* (by www. Bacteria in photos.com).

Staphylococcus aureus

Sthap A is a gram-positive coccus bacterium that is a member of the *Firmicutes*, and is frequently found in the nose, respiratory tract, and on the skin. It is often positive for catalase and nitrate reduction. Although *S. aureus* is not always pathogenic, it is a common cause of skin infections such as abscesses, respiratory infections such as sinusitis, and produces a toxin that causes food poisoning. Pathogenic strains often promote infections by producing potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies. Many studies demonstrate that *Staphylococcus aureus* Cowan I (SAC), a protein A-positive Staphylococcal strain is a potent and consistent inducer of IgM production by normal human peripheral blood mononuclear cells (Figure 31).

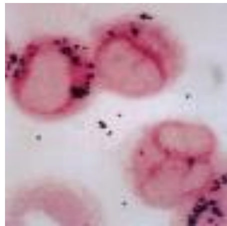


Figure 31: *Staphylococcus aureus.* Blackwell Science Ltd, Cellular Microbiology 1,101±117 (1999).

6.2-Aim of the study

Naïve B other cell activation is dependent on multiple signals initiated by binding of an Antigen (Ag) to the BCR. BCR cross-linking induces a signaling cascade leading to internalization and processing of the bound Ag, cytokines production, and up-regulation of costimulatory molecules. Other factor that strongly influence B cell activation are complement binding thorough the complement receptors CD21 as well as TLR interaction. In fact, a three-signal model for activation of human naïve cells has been suggested involving 1) BCR cross-linking, 2) Physical T-cells help, 3) TLR 9 stimulation and/or cytokines. Based on this issues, we focused attention to characterize the effects of different mucosal bacterial pathogens such as *Moraxella catarrhalis,* (Mcat), *Heamophilus influenzae type b (Hib), Haemophilus influenzae non typeable* (NTHi*), Staphilococcus aureus Cowan I (SAC)* on the isotype of antibody production from human B cells. The synthesis and secretion of immunoglobulins by Human Peripheral Blood Mononuclear cells (PBMCs) and Tonsils were evaluated by enzyme-linked immunosorbent assay (ELISA). Samples of cells actively secreting immunoglobulins and immunoglobulins quantification released into culture supernatant is a useful information in order to assess the Immunoglobulin class switch of B cells without T- cells help. In addition, flow cytometry analysis (BD FACSDiva 4.0) was performed to characterize the potential stimulation by surface bacterial protein before and after stimulation on mucosal B cell. The cells were stained with monoclonal antibodies to B cells markers in order to obtain a B-cells panel. Data were analyzed using FlowJo Softwere to evaluate the subpopulation of B-cells through the parental population of B-cells.

6.3-Materials and methods

6.3.1-Bacteria and culture conditions

Moraxella catarrhalis was clinically isolated as described in Mollenkvist et al., (2003). The bacterial strain was taken in sterile conditions from the Eppendorf tube. Two /three beds were placed into a blood agar petri dish and turned around and then wasted. The bacteria were grown on blood agar base solid medium at 37 C° 5% CO_2 over-night.

24 hours later in sterile conditions, one CFU (colony forming unit) was taken with a plastic stick and placed in 10 mLs of brain heart infusion (BHI) liquid medium.

Haemophilus influenzae type b (Hib) (ATCC 10211, Manassas, Virginia) and Haemophilus influenzae non-typeable (NTHi) (ATCC 49247) were rehydrated with 500 µLs tryptic soy broth. The whole content was aseptically taken and placed in 6 mL of TSB. Three drops have been placed in achocolate agar plate and incubated at 37° C (o/n). The suspension was spinned down (800 rpm 10' at RT) re-suspended in 1 mL of TSB +20% of glyceraldehyde and frozen at -80° C.

Serial dilution and plate count: *Moraxella* grew in 10mL of BHI. Six tubes, each with 9 mL of BHI, were settled up and six dilutions (1:10 until to $1:10^6$) were done. Some drops of each tube were placed in five petri blood agar plates and placed upside down inside the incubator at 37° C 5% CO_2.

Hib and NTHi were taken from the plate and diluted in 2 mL of BHI and diluted 6 times as described below (30µL of bacteria + 270µL PBS) and plated in chocolate agar plates. The bacteria were allowed to multiply for 24/48 hours.

6.3.2-Formaldehyde killing assay

Original bacteria tubes were spinned down and washed 3 times in phosphate buffer saline (PBS), at a pH of 7.2, by centrifugation. Thereafter they were suspended immediately in 0.5 % formaldehyde for 3 hours at room temperature followed by heat treatment at 80° C for 3 minutes. Formaldehyde stock at 10% was diluted to 1:20 (250 µL of PFA +4750 µL PBS) in 5 mL of PBS. After

being washed three times by centrifugation in PBS the bacteria were re suspended in RPMI-1640 (Gibco, Paisley, UK) and stored in five aliquots at -20'C. The CFUs of each bacteria strain were counted in order to do the stim assay.

6.3.3-Reagents

Protein A from *Staphylococcus aureus Cowan* I strain was purchased (P7155, Sigma Aldicrh, 3050 Spruce Street, St Louis, MO 63103 USA). Protein A is a highly stable cell surface receptor produced by several strains of *Staphylococcus aureus*. It consists of a single polypeptide chain with a molecular mass of 42 kDa, containing four repetitive domains rich in aspartic and glutamic acids, but devoid of cysteine. It contains little or no carbohydrate (Boyle and Reis, 1997). Protein A is capable to bind to the Fc portion of immunoglobulins, especially IgGs, from a large number of species. One protein A molecule has been shown to bind at least 2 molecules of IgG simultaneously (Sjöquist, 1972). The IgG binding domain of Protein A consists of three anti-parallel α-helicies, the third of which is disrupted when the protein is complexed with the Fc region of the immunoglobulin. Protein A will bind to the Fc portion of human IgG subclasses, IgM, IgA, and IgE; and mouse IgG1 (weakly), IgG2a, and IgG2b. Protein A also binds IgGs from other species, including monkey, rabbit, pig, guinea pig, dog, and cat (Lindmark, 1983). Protein A may be conjugated with various reporter molecules, including fluorescent dyes (FITC), enzyme markers (peroxidase, β-galactosidase, and alkaline phosphatase), biotin, and colloidal gold without affecting the antibody-binding site on the molecule. These conjugates are used to detect immunoglobulins in various immunochemical assays including Western blotting, immunohistochemistry, and ELISAapplications. In addition, Protein A may be immobilized on a solid support such as agarose or acrylic beads for the purification of either polyclonal or monoclonal immunoglobulins (Current Protocols in Immunology, Coligen et al., 1991).

CpG DNA ODN. (3950 Sorrento Valley Blvd, Suite 100 San Diego, California 92121 USA). Aliquots 1 mg/mL of CpG were stored at -80°. CpG ODNs are synthetic oligonucleotides that contain unmethylated CpG dinucleotides in

particular sequence contexts (CpG motif). These CpG motifs are present at a 20-fold greater frequency in bacterial DNA compared to mammalian DNA. CpG ODNs are recognized by Toll-like receptor 9 (TLR9) leading to strong immunostimulatory effects. Three classes of stimulatory CpG ODNs have been identified, classes A, B, C, which differ in their immunostimulatory activities. CpG strongly activate B cells but weakly stimulate IFN-α secretion.

Interleukin 21 (IL-21): This cytokine is produced primarily by T follicular helper cells, which support B cell differentiation, and greatly enhance proliferative responses and antibody production from purified human peripheral blood, tonsillar, and splenic B cells. Aliquots 100 µg/mL of IL-21 were stored at -80° C.

6.3.4-Culture cells and samples

Human peripheral blood cell

PBMCs from healthy donors were prepared by gradient centrifugation. Blood draw and cell isolation were approved by our local ethics committees. PBMCs were separated from 60 mL of fresh whole blood by centrifugation on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden). Lymphocytes of the buffy coat were washed in RPMI 1640 and the cell pellet was washed and finally resuspended in 'cellwash' medium (Becton Dickinson, Mountain View, CA) containing 0.5% (v/v) bovine serum albumin (cell wash/BSA). 17 samples were employed in the experimentation. Of these, 14 were frozen and three were fresh.

Tonsils.

Four patients with adenotonsillar hypertrophy who underwent tonsillectomy were enrolled. Tonsils were placed in ice-cold DPBS (1X) (GIBCO by Life technologies) plus antibiotics, and processing began <30 min after surgical excision. Tonsils were manually dissected and gently grounded with a syringe plunger through a 70-µ mesh screen. Lysis buffer removed red blood cells. Cellular viability was determined by trypan blue exclusion. Specimens with a viability <70% were discarded. In addition, two frozen samples of tonsils were employed (Table 35).

Frozen PBMCs	Fresh PBMCs	Frozen Tonsils	Fresh tonsils
14	3	2	4

Table 35: Samples enrolled during the experimentation.

6.3.5-B-cell stimulation (stimulation assay).

All stimulatory reagents were optimized for cell stimulation in the settings described (Table 36). PBMCs and Tonsils cells (1x10^6 cells/mL) in RPMI media (Invitrogen) with 10% heat-inactivated FCS (HyClone Laboratories) and 10mg/mL gentamicin (Invitrogen) were cultured in 96 well/plate in media alone or stimulated with IL-21 (10 ng/mL; Peprotech, Inc., Rocky Hill, NJ), CpG (1ug/mL, 3ug/mL, 10ug/mL; Integrated DNA Technologies, Inc. San Diego, CA), *Moraxella catarrhalis* (Mcat), *Heamophilus influenzae* type b (Hib), (ATCC 49247) and non typeable (NTHi) (ATCC 102111), at the final concentration of 10^5,$10^{5.5}$, 10^6, $10^{6.5}$ and *Staphilococcus aureus Cowan I* (SAC) (SIGMA) with a dilution factor from y_{20000} till y_{10^6}), at 37° C for three or five days. After three or five days the 96 wells plate was centrifuged at 12000 rpm for 10 min. The supernatant of cells was transferred into a new sterile 96 wells plate and analyzed by ELISAin order to establish the titer of Immunoglobulins, in particular IgGs and IgMs. The pellets were placed into tubes for FACS analysis.

Plate 1									
	PBMCs+ 100ul medium	PBMCs+MC1$0^5$+ medium	PBMCs+M.c1$0^{5.5}$+ medium	PBMCs+Mc10^6l+medium	PBMCs+Mc$10^{6.5}$+medium	PBMCs+HIb10^5+ medium	PBMCs+HIb$10^{5.5}$+l medium	PBMCs+HIb10^6+medium	PBMCs+HIb$10^{6.}$.5+ medium
	PBMCs +100ul medium	PBMCsl+MC 10^5+l medium	PBMCs+Mc1$0^{5.5}$+ medium	PBMCl+Mc10^6 +medium	PBMCs+Mc$10^{6.5}$+ medium	PBMCs+HIb10^5+medium	PBMCs+HIb$10^{5.5}$l+medium	PBMCs+HIb$10^{6.}$+medium	PBMCs+HIb$10^{6.5}$+medium
	PBMCs+ med+IL-21	PBMCsl+MC 10^5+ IL-21	PBMCs+Mc1$0^5$.5+ IL-21	PBMCs+Mc10^6+ IL-21	PBMCs+Mc$10^{6.5}$+ Il-21	PBMCs+HIb10^5+medium	PBMCs+HIb$10^{5.5}$+ medium	PBMCs+HIb10^6+ IL-21	PBMCs+Hib$10^{6.5}$+ IL-21
	PBMC+ med IL-21	PBMCs+MC1$0^5$+ IL-21	PBMCs+McC$10^{5.5}$+IL-21	PBMCs+Mc10^6+ IL-21	PBMCs+Mc$10^{6.5}$+ IL-21	PBMCs+HIb10^5+ IL-21	PBMCs+HIb$10^{5.5}$+IL-21	PBMCs+HIb10^6+ IL-21	PBMCs+HIb$10^{6.5}$+ IL-21
	200ul Medim								

Plate 2									
	PBMCs+ NTHi 10^5 +medium	PBMC+ NTHi $10^{5.5}$ + medium	PBMC+ NTHI10^6 +medium	PBMC+ NTHi $10^{6.5}$ +medium	PBMCs+ SAC +medium	PBMCs+ SAC +medium	PBMCs+ SAC +medium	PBMCs+ SAC +medium	PBMCs+ CpG +medium
	PBMCs+ NTHi 10^{5+} +medium	PBMC+ NTHi $10^{5.5}$ + medium	PBMC+ NTHi 10^6 +medium	PBMCl+ NTHi $10^{6\ 5}$ +medium	PBMCs+ SAC +medium	PBMCs+ SAC +medium	PBMCs+ SAC +medium	PBMCs+ SAC· +medium	PBMCs+ CpG+ medium
	PBMCs+ NTHi 10^5 +IL-21	PBMCs+ NTHi $10^{5.5}$ + IL-21	PBMCs+ NTHi 10^6 + IL-21	PBMCs+ NTHi 10^6 + IL-21	PBMCs+ SAC + IL-21	PBMCs+ SAC +IL-21	PBMCs+ SAC+ IL-21	PBMCs+ SAC + IL-21	PBMCs+ CpG + IL-21
	PBMCs+ NTHi 10^5 + IL-21	PBMCs+ NTHi10^5 .^{5} + IL-21	PBMCs+ NTHi 10^6 +IL-21	PBMCs+ NTHi 10^6 + IL-21	PBMCs+ SAC + IL-21	PBMCs+ SAC + IL-21	PBMCs+ SAC + IL-21	PBMCs+ SAC + IL-21	PBMCs+ CpG + IL-21

Table 36: Scheme of stimulation assay with two plate 1-2 in order to establish the best concentration of bacteria for using during experimentation. PBMCs were cultured with Mcat, Hib, NTHi, at the concentration of 10^5,$10^{5.5}$, 10^6, $10^{6.5}$ and SAC) (SIGMA) with a Df $y^{20000}$to $y_{10}{}^6$. CpG (10µg/mL) with or without IL-21 (10ng/mL).

6.3.6-Enzyme–linked immunosorbent assay (ELISA)

ELISA Total IgG and IgM antibodies were measured in the supernatant of cells following the manufacturer protocol. Briefly: 96 –well microtiter plates (NUNC Maxisorp; Thermo Fisher Scientific) were coated with capture antibody. IgM (Jackson Lab. 10902) diluted 1:12000; IgG (Jackson lab 116836) diluted 1:4000. Capture antibodies goat anti human were added to carbonate buffer (50mM) at the concentration mentioned before. 100µL of capture antibodies were placed in each well and incubated o/n. After 24 hours, the plates were washed (plate washer Skatron) three times with PBS and 200µL of PBS-t-BSA were placed in each well. The plates were incubated for 2 hours at room temperature. After 2 hours' immunoglobulins stocks Human IgM (Jackson 49215) and Human IgG (Jackson 80294 were diluted with PBS-t-BSA at the final concentration of 500 ng/mL. The plates were washed and 100 µL of PBS-t-BSA were added into wells A1-A2. 100 µL of standard were added simultaneously and mixed up and down at least 8 times. 100 µL of suspension was removed and through in B1-B2. The mix was repeat until H1-H2. This gives 1:2 dilutions. The practice was repeated adding 50 µL of samples; samples

give 1:3 serial dilution (Table 37). The plates were incubated for two hours at room temperature.

	Standard Final concentration	Samples diluition Dilution factor 1:3
A	250 ng/mL	Y_3
B	125 ng/mL	Y_9
C	62.5 ng/mL	Y_{27}
D	31.2 ng/mL	Y_{81}
E	15.6 ng/mL	Y_{243}
F	7.8 ng/mL	Y_{729}
G	3.9 ng/mL	Y_{2187}
H	1.95 ng/mL	Y_{6561}

Table 37: Serial dilutions for standards and samples applied in ELISA assays.

Unbound samples were removed and an anti-antibody molecule which is covalently bound to horseradish peroxidase (H_2O_2 oxidoreductase) labeled affinity- purified goat anti-human IgG and IgM (Jackson Laboratories, Bar Harbor, ME) were added to the wells. Conjugated antibody was added to PBS-t-BSA at the recommended dilution (record below in Table 38) and mixed thoroughly. The plates were washed using plate washer. 100 uL conjugate placed in each well and incubated one hours at room temperature.

Goat anti-human	Lot#	Dilution
M-HRPO	Jackson 49215	1:1000
G-HRPO	Jackson 96091	1:2000

Table 38: Dilutions of capture antibodies using in ELISA assays.

The detection of antibodies was performed by using the ABTS developer. The developer contains both the substrate (H_2O_2) and the chromogenic reactant ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)]/ H_2O_2. When H_2O_2 was degraded, the byproduct causes the chromogen to develop a green color. The amount of color formed is directly proportional to the amount of H_2O_2 degraded, which is directly proportional to the amount of anti-antibody–HRPO in the well, which is directly proportional to the amount of unknown bound in the well by the capture antibody. Concentrations were extrapolated from purified human IgG and IgM standards (Jackson Laboratories) bound to capture antibody that showed comparable slopes of binding Data were acquired

with the SoftMax program software and read with the VersaMaxPLUS ROMv1.21 at wavelength of 405 nm.

6.3.7 Statistics

Data were analyzed using the IBM SPSS Software (IBM Corporation 1 New Orchard Road Armonk, New York 10504-1722 United States). We have applied Non-Parametric Tests for Independent Samples, using the NPTESTS *post hoc tests* procedure for the Kruskal-Wallis omnibus test. Comparisons of antibody titers between groups were done using the Mann–Whitney test after base 10 logarithmic transformations. A $p< 0.05$ was considered to indicate statistical significance. In addition, the *one-way* analysis of variance (ANOVA) with a Dunnett's post *hoc test* in SPSS. The Dunnett's test is used when only the comparisons between treatments and a control group are of interest. Differences between groups were considered significant at a $p <0.05$.

6.3.8 Flow cytometer Analysis

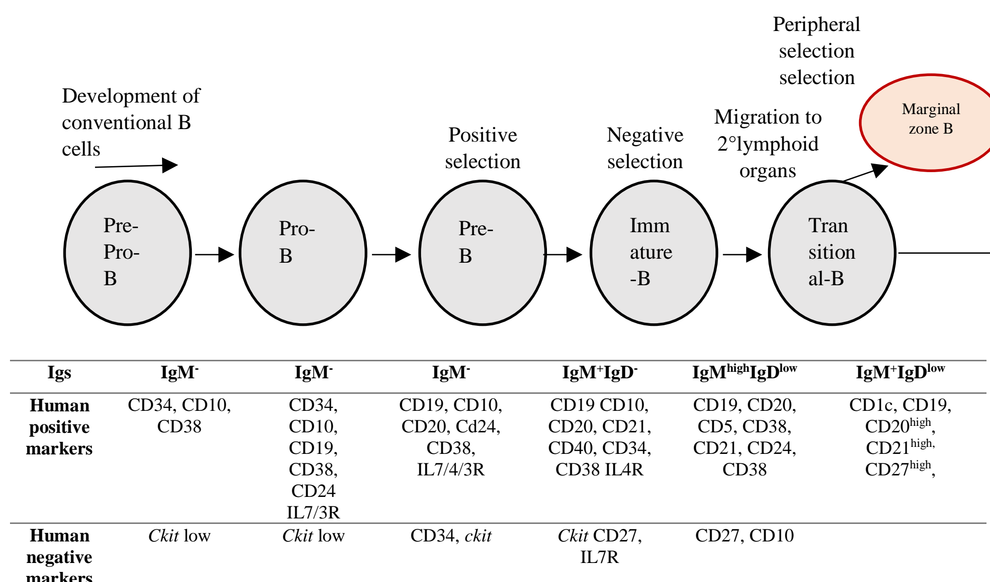
All samples (PBMCs and Tonsil cells) were stained with monoclonal antibodies to B cells markers (Table 39) (CD19-AF700, IgM-FITC, IgD-PE (Biolegend), CD21-PE Cy7, CD86 Pacific blue (BD Pharmingen) in order to evaluate the subsets, activation and responsiveness of B cells (switch class isotypes) after stimulation.

Marker	Fluor.	Vol (µl)	Marker	Fluor.	Vol (µl)
CD19	AF-700	2	**CD86**	Pacific-Blue	2
IgM	FITC	5	**Viability**	Amcyan	0.5
IgD	PE	5			
CD27	APC-H7	2			
CD21	PE-Cy7	2			

Table 39: Antibodies used in cells staining

The cells were washed in 40 mL of media, 40*xg* for 7 minutes. The supernatant was decanted and the pellet re-suspended by flicking the tube or by gentle vortexing. Samples were re-suspended in 5mL PBS and gently vortexed to create a homogenous cell suspension. 10 µL of the cell suspension was removed in order to have 10^6 cell/mL. Cells were stained for 30 min at room temperature. Subsequently they were washed twice and fixed in 1% paraformaldehyde for

10 minutes at 4°C. Data were acquired within 4 hours using a BD LSRII flow cytometer (BD Biosciences – Immunocytometry Systems, San Jose, CA). Data were analyzed using FlowJo Software (Tree Star Inc., Ashland,) based on B-cells subsets and markers (Figure 32)



Igs	IgM^-	IgM^-	IgM^-	IgM^+IgD^-	$IgM^{high}IgD^{low}$	IgM^+IgD^{low}
Human positive markers	CD34, CD10, CD38	CD34, CD10, CD19, CD38, CD24 IL7/3R	CD19, CD10, CD20, Cd24, CD38, IL7/4/3R	CD19 CD10, CD20, CD21, CD40, CD34, CD38 IL4R	CD19, CD20, CD5, CD38, CD21, CD24, CD38	CD1c, CD19, $CD20^{high}$, $CD21^{high,}$ $CD27^{high}$,
Human negative markers	*Ckit* low	*Ckit* low	CD34, *ckit*	*Ckit* CD27, IL7R	CD27, CD10	

Figure 32: Summary of the developmental stages and markers of B-cells

6.4-RESULTS AND DISCUSSION

6.4.1-Bacteria cultures

Moraxella catarrhalis was grown on brain heart infusion (BHI) agar plate and incubated at 37° C o/n (Figure 33). *Heamophilus influenzae* type b, *Heamophilus influenzae* non typeable were grown in chocolate agar petri dishes and incubated at 37° C o/n.

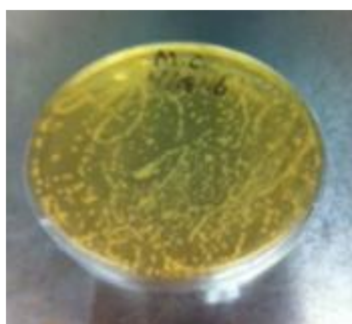


Figure 33: Colony Forming Unit of *Moraxella catarrhalis.*

CFUs of each bacterium were counted with program *Promega colony counter* (Promega Corporation Madison, WI 53711 USA). The number of bacteria for each strain is showed in Table 40.

Bacteria strain	Df	N of CFU	Total [bacteria]
Moraxella catarrhalis	1: 10^6	26	$26*10^6$
Haemophilus influenzae **(type b)**	1: 10^6	240	$240*10^6$
Haemophilus influenzae **(non typeable)**	1: 10^6	195	$196*10^6$

Table 40: CFUs obtained after 24 hours of growth at 37°C 5% CO2 of *Moraxella catarrhalis, Haemophilus influenzae* (type b), *Haemophilus influenzae (*non typeable).

6.4.2-Stimulation assay

Primarily we had to set up the optimal concentration of bacteria strain for its application in stimulation assays. *Moraxella catarrhalis*, Hib, NTHi, SAC, CpG, and IL-21 were cultured for five days with frozen PBMCs (1x10^6/mL) in two 96 well/plate as described before. An ELISA assay was performed in order to measure the IgGs titer in the samples.

PLATE#1: the amount of IgG of PBMCs after stimulation with media (control) and *Moraxella catarrhalis* (10^5 $10^{5.5}$ 10^6 $10^{6.5}$ concentrations) with and without IL-21 is shown in Figure 34.

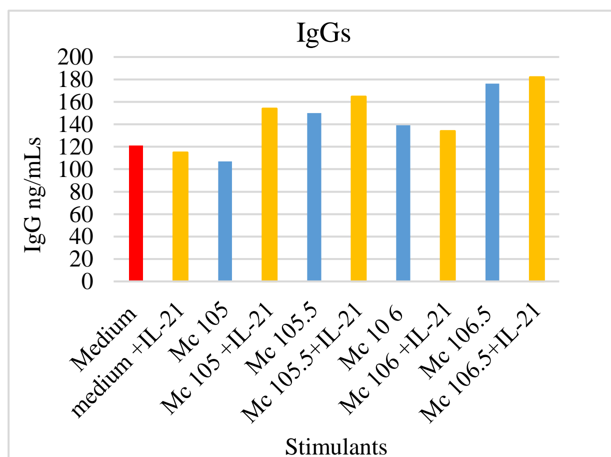


Figure 34: IgG titer of PBMCs after stim assay with *Moraxella catarrhalis*

We obtained optimal result with Mcat at the concentration $10^{6.5}$ with and without IL-21. Medium is the control, *Moraxella catarrhalis* have increased IgG production about of a 1.5 fold. In the past years the ability of *Moraxella* to

induce B cells activationhas been shown. It is also clear that *Moraxella* by means of the IgD binding protein MID induces a polyclonal B cells activation to promote a response, which is characterized by B cell proliferation and unspecific Igs production. (Singh at al., 2012). However, a low quantity of IgGs could be explained since after stimulation with Mcat. B cells need additional signals, (such as IL-4, IL-10 CD40L). In addition, most of data regarding the interaction of *Moraxella* with IgD^{+} B cells suggest that Mcat redirects the immune response by promoting the production of polyclonal low specificity Igs and thus results in avoidance of specific antibody response against the bacteria. Learning from *in-vitro* experiments with whole Mcat, it is evident that stimulation of BCR and TLR leads to initial IgM production but not in class switching *(*Souwer et al., 2009; Mollenkvist et al., 2003*).* On the other hand, if additional T_H2 cytokines are provided BCR cross-linking by MID induces class switching (Nordstrom et al., 2002).

In Figures 35 (A-B) were shown the results of ELISA performed with Hib and NTHi cultured with PBMCs.

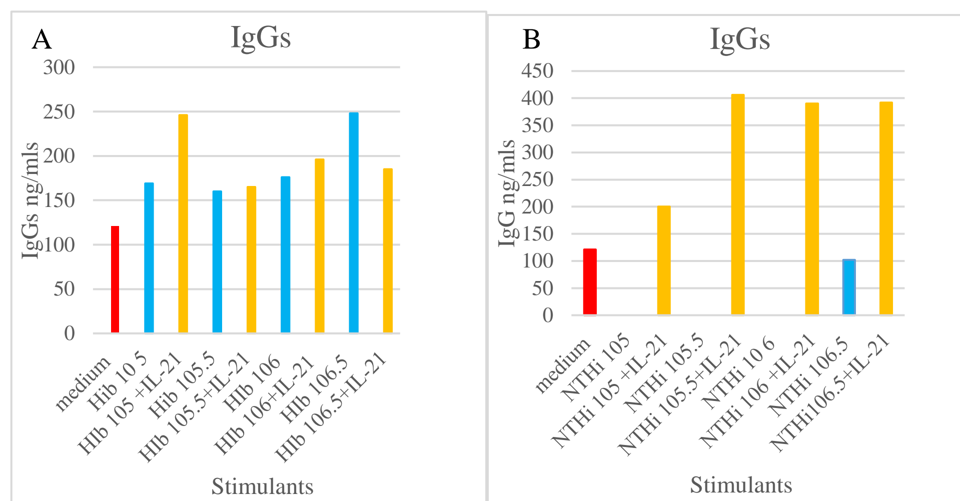


Figure 35 A: Titer of IgG obtained in stim assay with Hib and PBMCs after 5 days of culture. B: Titer of IgG obtained in stim assay with NTHi and PBMCs after 5 days of culture.

Antibodies against capsular polysaccharides (PSs) of bacteria, such as *Haemophilus influenza* type b (Hib), fix the complement to the bacterial surface and are protective in animals and humans. Capsular PSs, however, are T-independent type 2 antigens, do not induce immunologic memory, and yield isotype-restricted antibodies that mature late in ontogeny, so children <24

months of age do not make protective anti-PS antibody titers. The total ELISA IgGs measured in PBMCs after stimulation with Hib shows a better result with Hib 10^5 without IL-21 and Hib $10^{6.5}$ with IL-21. Hypogammaglobulinemia with IgG deficiency is the immune deficiency most clearly associated with NTHi infection (Van der Hilst et al., 2002). The administration of IgG replacement therapy has been shown to reduce systemic NTHi infections including a study which showed a reduction in nasopharyngeal colonization (with a possible effect from antibiotics as well) (Lindenberg et al., 2001). The data showed the best stimulation using NTHi $10^{6.5}$ with IL-21 (392 ng/mL)

In Figure 36, shown below, we have obtained 6160 ng/mL of IgGs after stimulation with SAC (y_{10^6}) + IL-21, 1091 ng/mL after stimulation with CpG (10ng/mL) and 1889 ng/mL of IgG after stimulation with CpG +IL-21 compared with a control medium 121ng/mL.

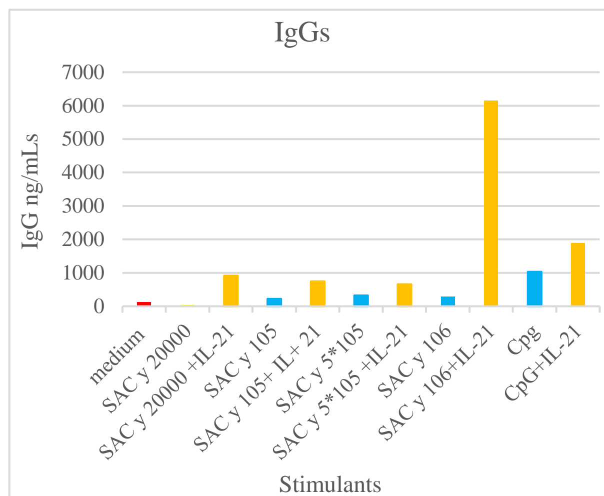


Figure 36: ELISA results obtained with SAC with a concentration range of (y_{20000} y_{10^5} y_{5*10^5} y_{10^6}), and CpG (10ug/mL).

Protein A is a 42 kDa surface protein originally found in the cell wall of the bacterium *Staphylococcus aureus*. It is encoded by the *spa* gene and its regulation is controlled by DNA topology, cellular osmolarity, and a two-component system called ArlS-ArlR. It has found use in biochemical research because of its ability to bind immunoglobulins. It is composed of five homologous Ig-binding domains that fold into a three-helix bundle. Each domain is able to bind proteins from many mammalian species, most notably IgGs. It binds the heavy chain within the Fc region of most immunoglobulins and within the Fab region in the case of the human VH3 family. Through these

interactions in serum, where IgG molecules are bound in the wrong orientation (in relation to normal antibody function), the bacteria disrupt opsonization and phagocytosis. IL-21 plays an important role in murine B cell differentiation into memory cells and plasma cells; we investigated the role of IL-21 in human B cell differentiation. In this study, we report that IL-21 co-stimulation not only is capable of inducing plasma cell differentiation from $CD27^{+}$ memory B cells, but also has the capacity to induce class switch recombination (CSR) and stimulate poorly responsive naive cord blood B cells into IgG-secreting plasma cells. Based on the ELISA performed with a concentration range of bacteria in order to achieved anboptimal stimulation of cells we used the concentrations reported in table 41.

Stimulant	Concentration in culture cells
Moraxella catarrhalis	$10^{6.5}$
Haemophilus influenza type b	$10^{6.5}$
Heamophilus influenzae non typeable	$10^{6.5}$
Staphilococcus aureus Cowan I	y_{10}^{6}
CpG	10ng/mL
IL-21	10ng/mL

Table 41: Optimal concentration of stimulants obtained from ELISA assays, used in next stimulations assays.

We applied the stimulants concentration reported in table 41 in order to measure the titer of IgM in PBMCs culture in the same conditions which are described previously. In Figure 37 the IgM ELISA assay result are showed.

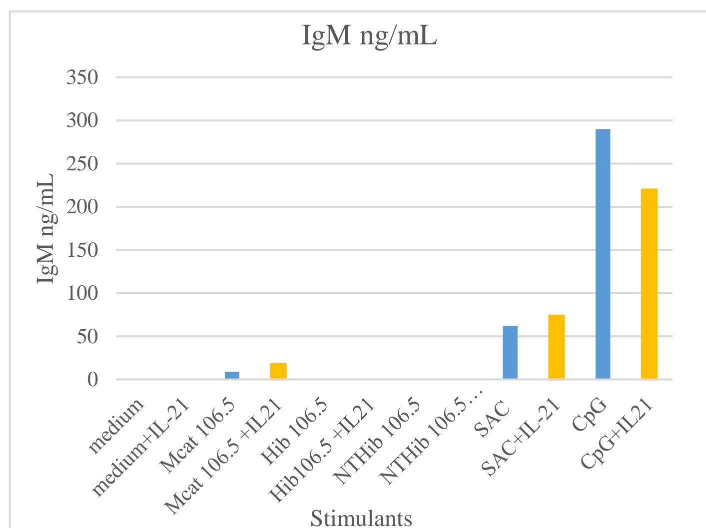


Figure 37: IgM titer in PBMC culture at days 5 of stimulation with bacteria, CpG and IL-21.

The IgMs baseline (medium) was undetectable as IL-21, Hib and NTHib. The higher concentration of immunoglobulins M was achieved with SAC (62ng/mL) and SAC+IL-21 (74ng/mL).

In Figure 38 (A-B-C-D), we showed the results obtained after five days of stimulations of frozen PBMCs in two subjects. In subject 5, the titer of IgG increased after culture with CpG+IL-21 (1389 ng/mL) compared to the medium (control) (89ng/mL). A slight increase was observed also with Mcat+ IL-21 and Hib+IL-21 (Figure 38-A). The titer of IgMs showed higher stimulation with CpG+IL-21. The production of IgM was about three time higher then IgG, these data suggest that the stimulants increase production of IgM into supernatants. These results indicate that naive B cells are activated to proliferate and to produce IgM by CpG ODN +IL-21 (Figure 38-B) On the hand the stimulation of IgG was not statistically different in subject 1, (Figure 38-C), but statistical different were IgMs after stimulation with SAC and CpG (3ug/mL) compared to control (medium).

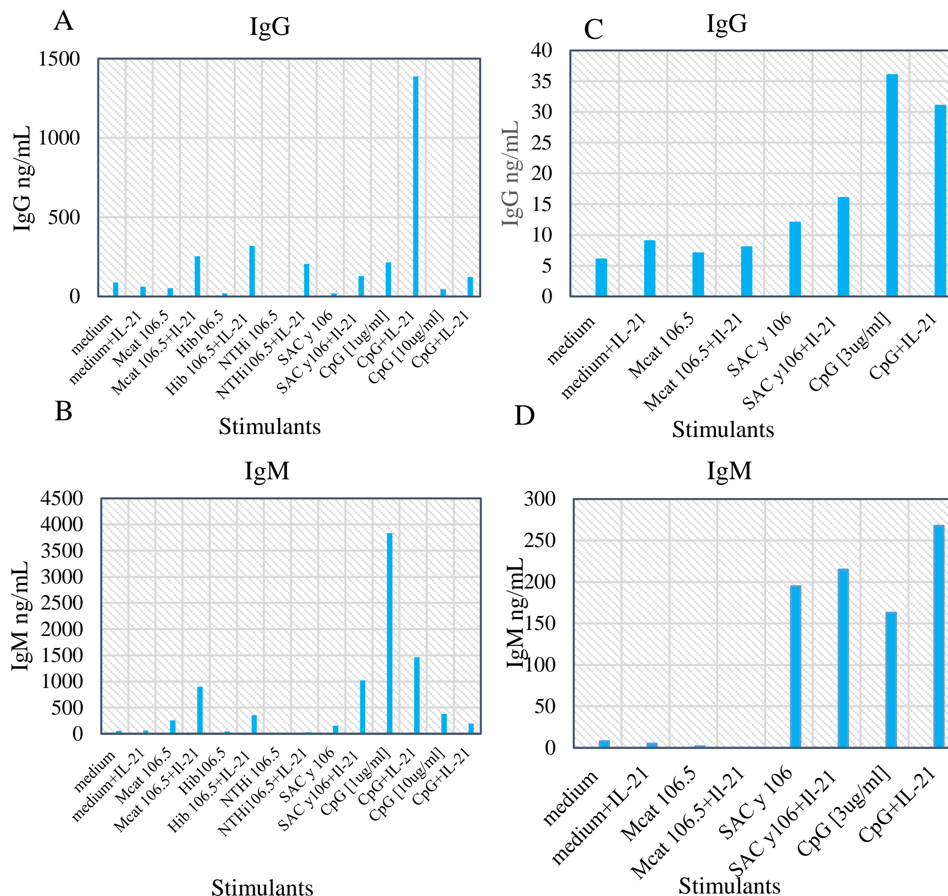


Figure 38: Titer of IgG and IgM of two samples (Patient ID5, and ID1) after 5 days of incubation with stimulants.

We considered also the production of IgG and IgM after five days' stimulation of fresh PBMCs and frozen tonsils (data no shown). In this assay no statistical differences were showed between control (medium and stimulants) nor in PBMC (*p-value* IgG, IgM; 0.429), neither in frozen tonsils (*p-value*= 0.443).

In all culture cells analyzed after five days of stimulation, no statistical differences were observed in production of IgG and IgM. The data obtained suggest that SAC increase the production of IgG better than IgM, on the other hand CpG increase the production of IgM rather than IgG (Figure 39).

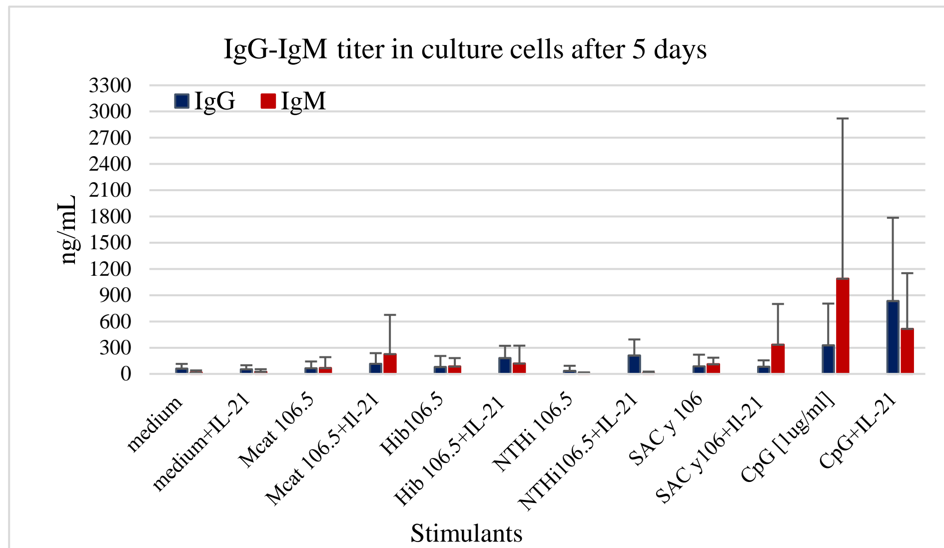


Figure 39: The histogram bars represent the average of IgGs and IgMs titer in cultured cells with medium (control) and stimulants ± standard deviation.

The data confirm the data observed by Jang et al. (2007). In addition, we have performed stimulation assays and ELISA assays after three days in order to compare the titer of IgG and IgM in the supernatants.

In Figure 40 ELISA results are shown after three days of stimulation in the same conditions mentioned before. We obtained stimulation of IgG by all stimulants; however, CpG and CpG+IL-21 showed a higher production of IgG in all cell cultures. IgM stimulation was higher than IgGs and again CpG and CpG +IL-21 showed the best results.

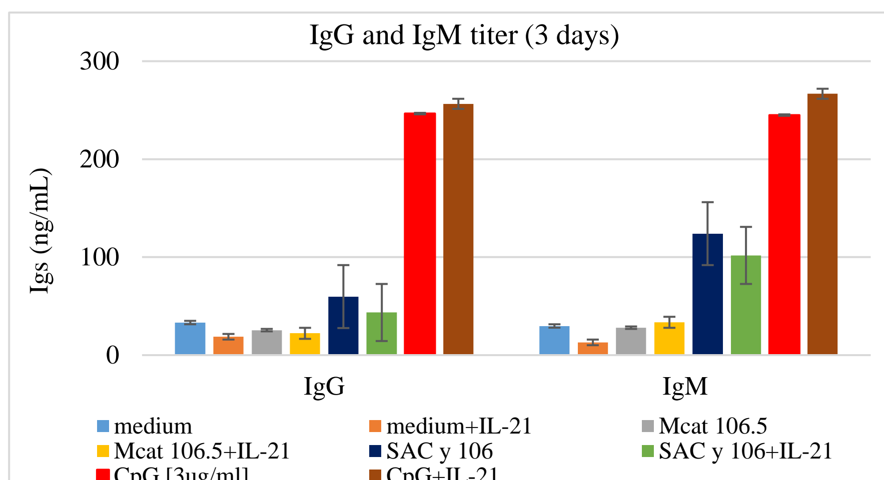


Figure 40: IgM and IgG titer in supernatant of culture cells obtained after three days of stimulation, Bars represent mean and SEM of samples analyzed

We performed statistical analysis on the production of IgGs and IgMs by different cultured cells after three days of stimulation with selected bacteria, IL-21 and CpG (Table 42).

Stimulant	Mean IgG ±SEM	Mean IgM±SEM
Medium	33.25 (34.25)	29.75 (50.2)
Medium+IL-21	18.75 (13.8)	13 (17)
Mcat	25.5(22.3)	2 (27.7)
Mcat+IL-21	22.2(24.3)	33.5 (29.5)
SAC	59.7 (88)	124 (89.4)
SAC+IL-21	43.5 (61)	101.7 (92.8)
CpG	245 (346)	245 (313.06)
CpG+IL-21	256 (347)	266.7 (325)

Table 42: Mean of values of IgG, IgM into supernatants after three days of stimulation

Figure 41 showed the graph of IgG mean quantity produced by cells after three days of stimulation. Higher stimulation was observed in the supernatants of cells stimulated with CpG and CpG+IL-21. However, the difference of titer of IgG among the stimulants was not statistically different.

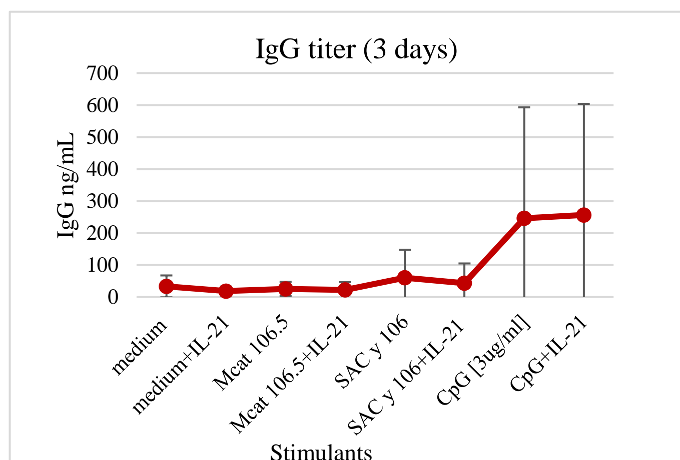


Figure 41: The graph showing the type of stimulant (Medium, medium+IL-21, Mcat, Mcat+IL-21, SAC, SAC+IL-21, CpG, CpG+IL-21) X-axis, against the mean quantity of immunoglobulins G (y-axis).

The same approach was performed with the data of IgM titer (Figure 42). In addiction the production of IgMs into the supernatant after three days resulted statistically different. We applied Dunnet T test for multiple comparisons, the data obtained showed that the titer of IgMs after stimulation with CpG +IL-21 was statistically different compared to the medium (*p-value*=0.037), IL-21 (*p-value*=0.023), Mcat (*p-value*=0.032), andMcat +IL-21 (*p-value*=0.003). The differences among other stimulants were not statistically different.

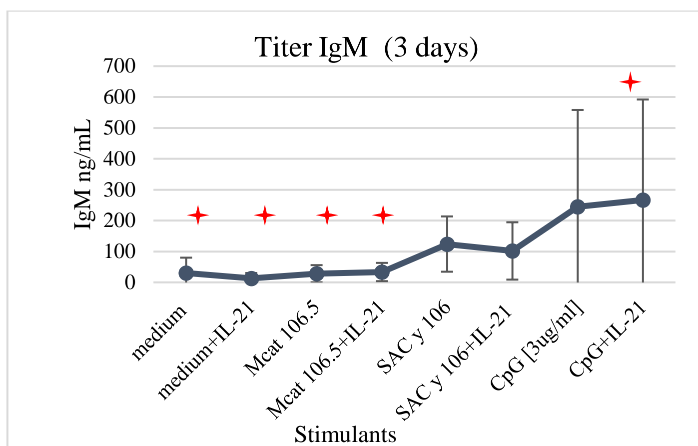


Figure 42: The graph showing the type of stimulant (Medium, medium+IL-21, Mcat, Mcat+IL-21, SAC, SAC+IL-21, CpG CpG+IL-21) X-axis, against the mean quantity of immunoglobulins M (y-axis). +*P-value<0.05.*

The data confirmed those obtained after five days of stimulation with CpG+IL-21.

6.4.3-Flow Cytometer Analysis

All samples (PBMCs and Tonsil cells) were stained with monoclonal antibodies to B cells markers as previously described. In all samples cells (PBMCs fresh and frozen, tonsils fresh and frozen) the distribution of circulating B-cells subsets, as defined in table 43.

Surface Markers Defining 10 B cell Subssets					
B cell Subset	**IgD**	**IgM**	**CD27**	**CD21**	**CD86**
Naïve/Transitional	+	+	-	↓	↑
IgM Memory	-	+	±	↓	↑
BND anergic	+	-	-	↓	↑
IgD Memory	+	-	+	↓	↑
Class Switch Memory (IgG, A, E)	-	-	±	↓	↑

Table 43: Surface Markers Defining B Cell Subsets IgM memory and class switching memory are each divided into two subsets based on CD27 expression

B cells are identified among PBMC based on initial selection of lymphocytes by forward and side scatter, selection of singlets (FSC-W vs FSC-H), and fating on CD19^{+}cells (live cells).

In addition, the subsets were identified through Boolean gating with anti-IgD, IgM, and CD27. Activation is determined by a lack of expression of CD21 and expression of CD86 on each subset. In Figure 43 a population of PBMC cultured in medium (control) is shown.

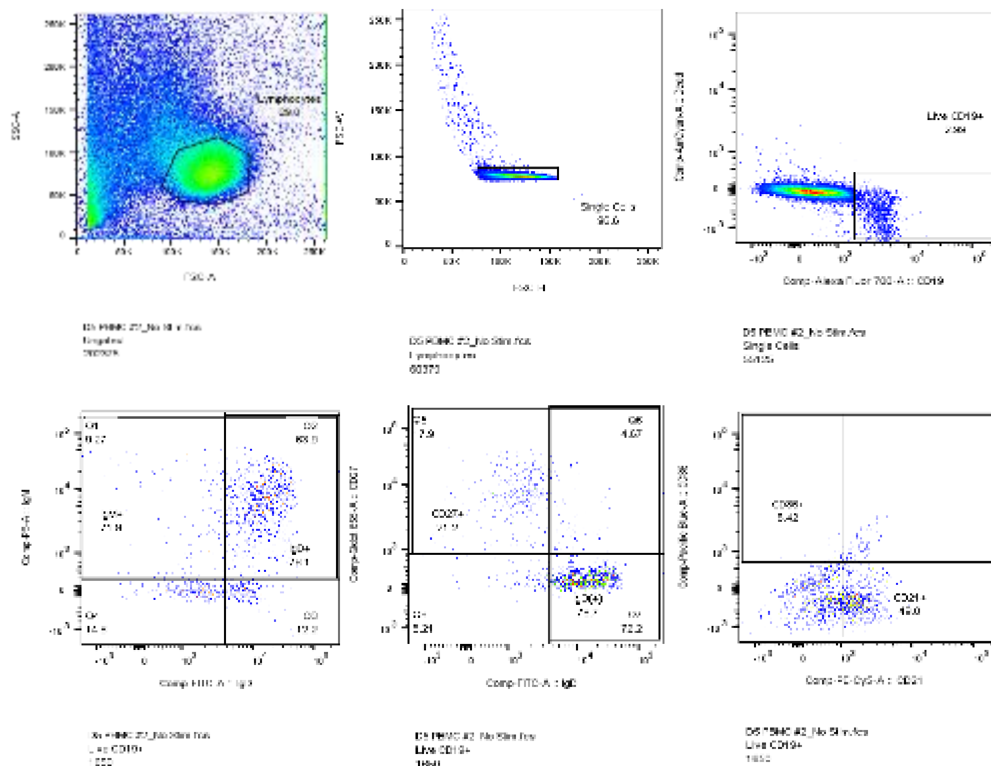


Figure 43: Gating scheme for CD19^{+} B cell subset of PBMC at baseline.

At day 5 in PBMC culture control and culture with stimulants naïve B cells (CD27-IgD^{+}) represented a comparable majority of the B cells in all samples analyzed. The membrane form of Ig is expressed on the surface of B cells in

the bone marrow. Stimulants, in particular SAC, SAC+IL-21 and CpG+IL-21 through the BCR not only steer the B cells through development but also allow to express a second isotype followed by class switch memory ($IgD^- IgM^-$) in medium but no in cultures with stimulants (Figure 44). In both cases (medium and stimulants) the majority of classes switch memory cells expressed CD27. (Memory cells $CD27^+ IgD^-$). Another subset of IgD^+ B cells, circulating B_{ND} ($IgD^+ IgM^- CD27^-$), considered to be both autoreactive and typically anergic, were significantly prevalent in all cultured cells with stimulants. IgM class switch memory ($IgD^- IgM^+$) is a subset of B cells that contains high secretion in response to T independent antigen.

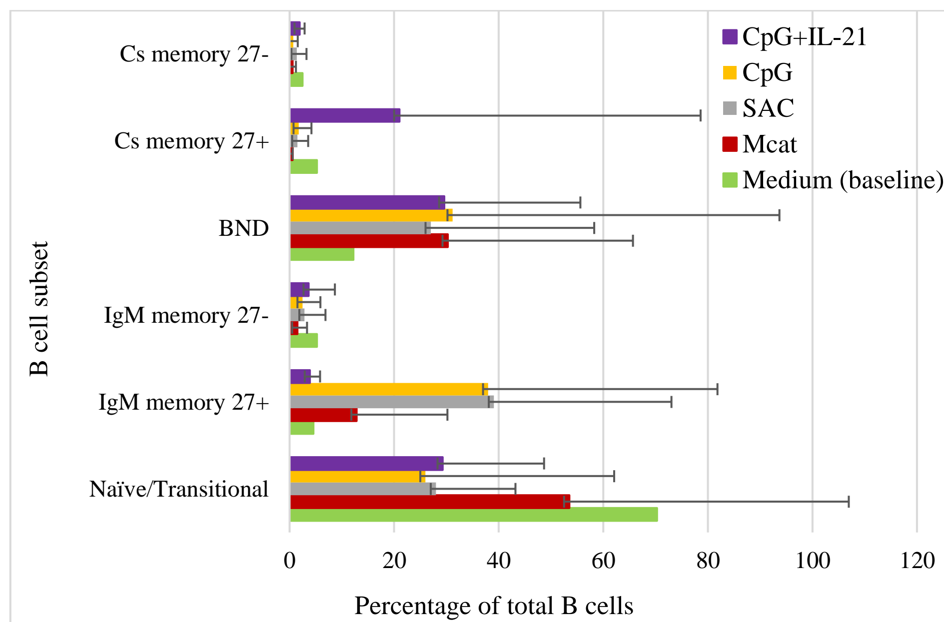


Figure 44: Distribution of B cell subset in cultures cell after 5 days stimulated with medium (control) and stimulated with Mcat, SAC, SAC+IL-21, CpG, CpG+IL-21.

By using Mann-Whitney non-parametric test that compares the ranks of two independent samples we didn' observe any statistical difference regarding the B cell subset between cultured cell with medium and cultured cells with stimulants.

<u>B cell subsets in response to stimulation *in-vitro*</u>

An essential element of the B cells function is their ability to be activated by exogenous and endogenous stimuli. As mentioned before, we focused our attention to characterize the effects of different mucosal bacterial pathogens on the isotype of antibody production from human B cell. This potential "decoy"

stimulation by surface bacterial protein that are proposed to stimulate B cells non-specifically by cross-linking surface antibody by the constant Fc region rather than binding these bacteria by the antigen-specific Fab variable region. In PBMC cultures, stimulation *in-vitro* with Mcat, Sac, CpG and IL-21, was associated with a lower density of CD21 and a higher density of CD86, on the cells surface. The magnitude of stimulation of PBMC was compared whit PBMC culture with medium only, (control) (see Figure 45).

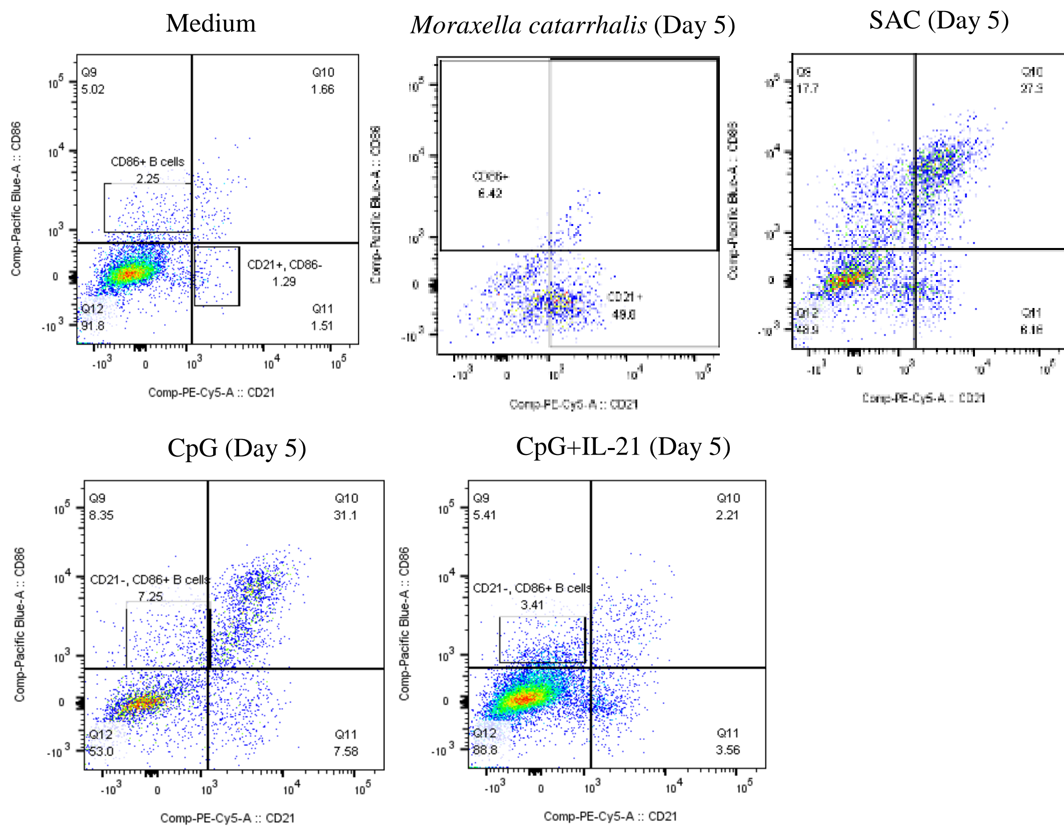


Figure 45: FlowJo analyses after 5 days of stimulations (medium, Mcat, SAC, CpG, CpG+IL-21).

The gating scheme for CD21 and CD86 comparing representative data from a representative PBMC culture in medium and PBMC culture whit Mcat, SAC, CpG and CPG +IL 21is shown. The percentage of $CD19^{+}$ B cells expressing CD21 and CD86 was compared among PMBC cultures with medium and PBMC culture cell stimulated with Mcat SAC, CpG, CpG+IL-21. Values are shown for the mean and standard deviation (Figure 46).

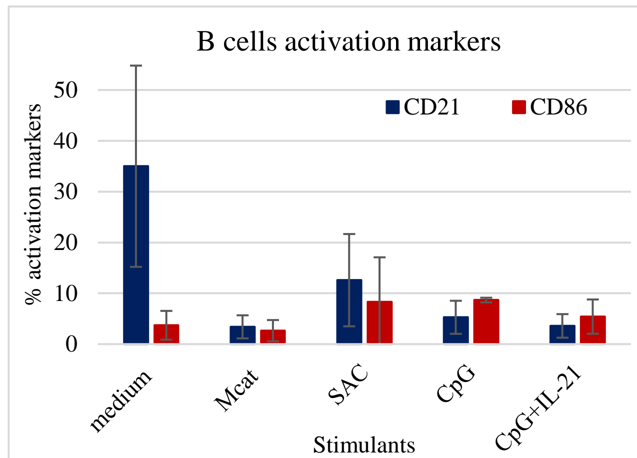


Figure 46: B cells activation markers CD21 (blue bars) CD86 (red bars).

Groups were compared through one-way ANOVA with the Dunett *post-hoc* test. As shown in Figure 20, the expression of CD21 decreases in cell cultures stimulated for 5 days. However, no statistical difference was observed in the CD21 expression between medium and stimulants. The expression of CD86 was statistically different among the groups (*p-value*=0.027). In particular, significant differences were observed between medium and SAC (*p-value* 0.0214) and medium and CpG (*p-value*= 0.0123) and between Mcat and SAC (*p-value*=0.0129) and Mcat and CpG (*p-value* =0.0123). Compared to other stimulants CpG and SAC elicited *in-vitro* activation of B cell after 5 days of stimulation. Expression of CD86 were less prominent in stim culture whit Mcat and CpG+IL-21. Indeed, despite differences between medium and stimulants the magnitude of change (percentage expression) of CD21 and CD86, activation markers in PBMC population was not statistically significant for all stimulants. CpG + IL-21 and Mcat probably required other molecules to activate B cell differentiation.

6.5-CONCLUSIONS

We identified B cells subsets and activation after stimulation with different nasopharyngeal and lung infected bacteria, CpG DNA, and Interleukin-21 (IL-21) compared with those in control subset, after 3 or 5 days. IgG ELISA assays were performed to identify the optimal concentration to use in the subsequent stimulation experiment. CpG + IL-21 enhances the production of immunoglobulins (IgG, IgM). CpG dinucleotides induce B cells to proliferate

and secrete immunoglobulins, without T-cells help. IL-21 is a pleiotropic cytokine, whose action generally enhances antigenic-specificity responses in immune cells; nevertheless, it requires T-cells help. We have applied the optimal concentration of stimulant in order to assess immunoglobulins production in 23 samples composed by frozen PBMC, fresh PBMC frozen tonsils and fresh tonsils. In all samples, the data showed higher increase of IgGs and IgMs after stimulation by SAC, CpG +IL-21. In particular, the magnitude change of IgGs production was on average 2071 ng/mL of IgG production after stimulation by SAC and on average 1917 ng/mL of IgM production after stimulation by CpG+IL-21. The results were confirmed in samples stimulated three days in the same conditions. On the other hand, IgMs production was higher after stimulation with CpG and IL-21. Cultures set up with B cells of healthy donors produced large amounts of IgM even in the absence of T cells stimulated by CpG +IL-21. Compared with stimulation by only medium Mcat influenced the serum levels of IgG or IgM in frozen PBMC but no in tonsils cells. However, no statistical difference was observed in samples, Hib and NTHi failed to produce significant levels of IgG, and IgM. Bacterial pathogens are not sufficient to induce class switching. The mechanism to account for co-colonization of bacterial pathogens influencing the host adaptive immune responses may involve activation and crosstalk of host multiple pattern-recognition receptors (PRRs) in innate immune cells, and components bridging innate and adaptive immunity. The host innate immune system detects microorganisms and responds to their stimuli mainly through Toll-like receptors (TLRs) (Kawai and Akira, 2005). Hib and Mcat can be recognized by TLR-2, 4 and 9 (Vuononvirta et al., 2007; Hassan et al., 2012; Clemente et al., 2012) found that the innate immunity induced by NTHi can provide protection against the *Streptococcus pneumoniae* (Spn) infection in mouse lung, probably through multiple innate immune mechanisms including formyl peptide receptors, complement receptors, and TLRs. Lim et al. (2008) reported that *Spn* synergizes with NTHi to induce inflammation via up-regulating TLR2. Spn and Hib synergistically activate NF-κB as well as the subsequent inflammatory TNFα, IL-1β, and IL-8 responses via IKKβ-IκBα and p38 MAPK pathway (Kweon et al., 2006). Such effects of co-colonization by Spn with Hib on innate immune immunity may lead to influence adaptive immunity.

Dendritic cells (DC) play important roles in bridging innate and adaptive immunity (Novak et al., 2010). TLRs and cross-talk between TLRs, and complement receptors in antigen present cells (APC) control adaptive immune responses (Pasare et al., 2005). TLR signaling not only helps to initiate adaptive immune response, but also is required for it (Schnare et al., 2001; Medzhitov et al., 2002). Activation of TLR in DCs results in elevated levels of pro-inflammatory cytokines or chemokines leading to the induction of the adaptive immune response, where both T and B-lymphocytes play a crucial role (Majewsaka et al., 2006). We observed that after three days of stimulation by CpG+IL-21 the magnitude of change of IgMs into the supernatant was statistically different compared to the medium, IL-21, Mcat, but not statistically different compared to the SAC.

We found that the majority of B cell subsets were similar between medium (control) and stimulated culture cells. However, we did not confirm the increased frequency of class switch memory B cells in stimulated cultures. It worth to mention that a higher percentage of IgM Memory B-cells in stimulated culture with SAC, SAC+IL-21, CpG+Il-21 compared to the medium. Immunological memory is our reservoir of ready-to-use antibodies and memory B-cells. Because of the immunological memory, a secondary infection will be very light or not occur at all. Antibodies and cells, generated in the germinal center in response to the first encounter with antigen, are highly specific, remain in the organism virtually forever and are mostly of IgG isotype. Long-lived plasma cells homing to the bone marrow ensure the constant production of protective antibodies, whereas switched memory B cells proliferate and differentiate in response to a secondary challenge. IgM memory B cells represent our first-line defense against infections. They are generated by a T-cell independent mechanism probably triggered by Toll-like receptor-9. They produce natural antibodies with anti-bacterial specificity and the spleen is indispensable for their maintenance. IgM memory B-cells have a great importance in the immediate protection against pathogens. The more mature class switch memory cells were lower in all stimulated cells compared to the medium. Among the minority B cells subsets, the frequency of B_{ND} cells was similar in cells culture in medium as well as cell cultured with stimulants.

Studies in human subjects have demonstrated that a substantial frequency of newly generated B cells was autoreactive. Deficiency of proper B-cell tolerance contributes to the development of autoantibodies, leading to initiation and perpetuation of autoimmune processes. At least three main mechanisms accounting for silencing of self-reactive B cells are known: receptor editing, deletion, and anergy. The process of anergy is an important tolerance mechanism whereby cells are functionally inactivated. Anergic B cells are characterized by a reduced ability to proliferate and secrete antibodies on antigens encounter accompanied by reduced BCR-signaling responses, including intracellular calcium mobilization and tyrosine phosphorylation (Szodoray et al., 2016). However, the stimulation condition used in our study may not have been of sufficient potency or duration to induce these B_{ND} cells to differentiate *in vitro*. Almost all B cell subsets, other than B_{ND}, showed changes with stimulation. These data supports the hypothesis that, Mcat SAC, CpG and IL-21, using appropriate conditions could induce the class-switching isotype without T-cell help. Post stimulation, expression CD21 decreased and CD86 increased compared to the control. For the future, we will investigate intracellular AID protein concentration. AID is required for SHM, which drives antibody affinity and function. Potential limitations of this study include a small sample size. We will enroll higher number of patients. In addiction based on these issues, we could investigate *Streptococcus pneumoniae* (Spn), *Haemophilus influenzae* (Hi) and *Moraxella catarrhalis* (Mcat), the most common pathogenic bacteria of respiratory infections and common commensal microbes in the human nasopharynx (NP). The effect of interactions among bacteria during co-colonization of the NP on the host immune response has not been well evaluated. The objective of future study could be to assess the impact of co-colonization by Hi or Mcat on the systemic antibody response and to vaccine protein candidate antigens and similarly, the impact of co-colonization by Spn and Mcat on antibody responses to Hi vaccine protein candidate antigens.

7-REFERENCES

1) Abiri, R., Valdiani, A., Maziah, M., Shaharuddin, NA., Sahebi, M., Yusof, ZN., Atabaki, N., Talei, D. 2016. A Critical Review of the Concept of Transgenic Plants: Insights into Pharmaceutical Biotechnology and Molecular Farming Curr Issues Mol Biol.; 18:21-42.

2) Adams, CA. 2006. Nutrition-based health in animal production. Nutrition Research Reviews. 19:79–89.

3) Aguirreburualde, MSP., Gómez, M., Ostachuk, A., Wolman, F., Albanesi, G., Pecora, A., Odeon, A. 2013. Efficacy of a BVDV subunit vaccine produced in *alfa-alfa* transgenic plants. Vet. Immunol. Immunopathol. 151, 315–324.

4) Ahmad, N., Michoux, F., Lossl, A., Nixon, P. 2016. Challenges and perspectives in commercializing plastid transformation technology. J. Exp. Bot. doi: 10.1093/jxb/erw360.

5) Ahmed, A., Broides, A., Givon-Lavi, N., Peled, R., Dagan, R., Greenberg, d.2008. Clinical and laboratory aspects of *Moraxella catarrhalis* bacteremia in children. Pediatr Infect Dis J 27, 459-461

6) Albert, H., Dale, EC., Lee, E., Ow, DW. 1995. Site-specific integration of DNA into wild-type and mutant *lox* sites placed in the plant genome. Plant J.; 7:649–659.

7) Allen, GC., Hall, G., Michalowski, Jr S., Newman,W., Spiker ,S. 1996. High level transgene expression in plant cells: effects of a strong scaffold attachment region from tobacco. Plant Cell, 8(5):899–913.

8) An, YQ., Huang, S., McDowell, JM.,. McKinney, EC., Meagher, RB. 1996. Conserved expression of the *Arabidopsis* ACT1 and ACT3 actin subclass in organ primodia and mature pollen Plant Cell, 8 (1) 15–30

9) Andrews, LB., Curtis, WR. 2005. Comparison of transient protein expression in tobacco leaves and plant suspension culture. Biotechnolo. Prog. 21,946-952.

10) Andrews, LB., Curtis, WR. 2005. Comparison of transient protein expression in tobacco leaves and plant suspension culture. Biotechnolo. Prog. 21,946-952.

11) AOAC (1998). Official Methods of the American Oil Chemists Society. American Oil Chemists Society, 5th ed. Champaign, Ill.

12) AOAC (2005). Official Methods of Analysis. Association of Official Analytic Chemists 18th ed. Gaithersburg, MD, USA.

13) Arango, IP., Rubio, E.L., Anaya, ER., Flores, TO., De la Vara, LG., Lim, MG. 2008. Expression of the rabies virus nucleoprotein in plants at high-levels and evaluation of immune responses in mice. Plant Cell Rep. 27, 677–685.

14) Arntzen, C. 2015. Plant-made pharmaceuticals: from 'Edible Vaccines' to Ebola therapeutics. Plant Biotechnology Journal. 13:1013–1016.

15) Arntzen, CJ. 2015. Plant-made pharmaceuticals: from 'Edible Vaccines' to Ebola therapeutics.

16) Bajaj, S., Puthigae, S., Templeton, K., Bryant, C., Gill, G., Lomba, P., Zhang, H., Altpeter, F. Hanley, Z. 2008 Towards engineering drought tolerance in perennial ryegrass using its own genome. Sixth Canadian plant genomics workshop.

17) Banda, R., Yambayamba, V., Lalusha ,BD., Sinkala., E., Kapulu, MC., Kelly, P. 2012. Safety of live, attenuated oral vaccines in HIV-infected Zambian adults: oral vaccines in HIV. Vaccine. 17;30(38):5656

18) Barakat, A., Gallois, P., Raynal, M., Mestre-Ortega, D., Sallaud, C., Guiderdoni, E., Delseny, M., Bernardi, G. 2000. The distribution of T-DNA in the genomes of transgenic Arabidopsis and rice. FEBS Letters. 471:161-164.

19) Barta, A., Sommergruber, K., Thompson, D., Hartmuth, K., Matzke, MA., Matzke, AJ. 1986. The expression of a nopaline synthase - human growth hormone chimeric gene in transformed tobacco and sunflower callus tissue. Plant Mo.l Biol.; 6(5):347-57.

20) Belanger, FC., Kriz, AL. 1989. Molecular characterization of the major maize embryo globulin encoded by the *Glb1* gene. Plant Physiol., 91, 636–643.

21) Bianchi, ATJ., Scholten, JW., van Zijderveld, AM., van Zijderveld, FG. 1996. Parenteral vaccination of mice and piglets with F4+ 683 *Escherichia coli*

suppress the enteric anti-F4 response upon oral infections. Vaccine; 14:199–206.

22) Bock, R. 2015. Engineering Plastid Genomes: Methods, Tools, and Applications in Basic Research and Biotechnology. Annu. Rev. Plant. Biol. 66:211–41.

23) Boothe, J., Nykiforuk, C., Shen, Y., Zaplachinski, S., Szarka, S., Kuhlman, P., Murray, E., Douglas, M., Moloney, M. 2010. Seed-based expression systems for plant molecular farming. Plan. Biotech Jour. Vol 8, Issue 5 588–606.

24) Boudry, G., Guérin, S., Henri Malbert, C. 2004. Effect of an abrupt switch from a milk-based to a fiber-based diet on gastric emptying rates in pigs: difference between origins of fibre. Br J Nutr.;92(6):913-20.

25) Boyle, MDP., Reis, KJ. 1997. Bacterial Fc Receptors. Biotechnology, 5, 697-70 3.

26) Boynton, JE., Gillham, NW., Harris, EH. 1988. Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science 240, 1534–1538.

27) Brandtzaeg, P. 1987. Immune function and immunophatology of the palatine and nasopharyngeal tonsils, immunology of the ear 63-106. New York Raven press.

28) Brandtzaeg, P. 2002. Current understanding of gastrointestinal immunoregulation and its relation to food allergy. Ann. N.Y. Acad. SCI. 964, 13-4.

29) Brandzaeg, P., Halstensen, TS. 1992 Immunology and immunopathology of tonsils. Adv Othorinolaringol.47, 64-75.

30) Brantzaeg, P., Baekkevold, ES., Farstad, IN., Jahnsen, FL., Johansen, FE., Nilsen, E., Yamanaka, T. 1999b. Regional specialization in the mucosal immune system; what happens in the micro compartments? Immunol. Today 20, 141-151.

31) Brogden, KA. 2000. Bacterial evasion of host –derived antimicrobial peptides on mucosal surface. Virulence mechanism of bacterial Pathogens (E.d. wannemuekler). Washington, DC: ASM Press;19-40.

32) Busch, A., Waksman, G. 2012. Chaperone-usher pathways: diversity and pilus assembly mechanism. Philos Trans R Soc Lond B Biol Sci 367:1112–1122.

33) Casellas, R., Nussenzweig, A., Wuerfell, R., Pelanda, R., Reichlin, A., Suh, H., Qui, X., Beser, E., Kenter, A., Rajesky, K., Nussenzweig, M. 1998. Ku80 is required for immunoglobulin isotype switching. EMBOJ. 17, 2404-2411.

34) Catrice, EV., Sainsubury, F. 2015. Assembly and purification of polyomavirus –like particles from plants. Mol. Biotechnol. 57, 904-913.

35) Cebra. J., Periwal, S., Lee, G., Lee, F., and Shroff, K. 1998. Development and maintenance of the gut associated lymphoid tissue (GALT) the roles of enteric bacteria and virus. Dev Immunol. 6, 13-18.

36) Chappuis, G. 1998. Neonatal immunity and immunisation in early age: lessons from veterinary medicine. Vaccine. 16:1468–1472.

37) Chavez-Barcenas, AT., Valdez-Alarcon, JJ., Martinez-Trujillo, M., Chen, L., Coconostle- Cazares, B., Lucas WJ., Herrera-Estrella, L. 2000. Tissue-specific and developmental pattern of expression of the rice sps1 gene. Plant Physiol., 124, 641- 654.

38) Chawla, R., Ariza-Nieto, M., Wilson, AJ., Moore, SK., Srivastava, V. 2006. Transgene expression produced by biolistic-mediated, site-specific gene integration is consistently inherited by the subsequent generations. Plant Biotechnol J 4:209–218.

39) Chen, Y., Zhao, L, Cao, Y., Kong, X., Hua, Y. 2014. 24 kDa and 18 kDa oleosins are not only hydrolyzed in extracted soybean oil bodies but also in soybean germination. J. Agric. Food Chem. 62, 956–965.

40) Chen, ZL., Schuler, MA., Beachy, RN. 1986. Functional analysis of regulatory elements in a plant embryo-specific gene. Proc Natl Acad Sci U S A, 83 (22); 8560–856.

41) Christensen, H., Sharrock RA., Quail PH. 1992. Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation Plant Mol Biol, 18 (4);.675–68.

42) Christian, M., Cermak, T., Doyle, EL., Schmidt, C., Zhang, F., Hummel, A. 2010. Targeting DNA double-strand breaks with TAL effector nucleases. Genetics 186:757–76.
43) Clemente, M., Corigliano, MG. 2012. Overview of plant-made vaccine antigens against malaria. J Biomed Biotechnol, 10, pp. 1–8.
44) Cockerill, P.N., Garrard, T. 1986a. Chromosomal loop anchorage sites appear to be evolutionarily conserved. FEBS Lett. 204,5-7.
45) Coddens, A., Diswall, M., Angström, J., Breimer, ME., Goddeeris, B. 2009. Recognition of blood group ABH type 1 determinants by the FedF adhesin of F18-fimbriated *Escherichia coli*. J Biol Chem 284:9713–9726.
46) Conrad, U., Fiedler, U. 1998. Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity. Plant Mol. Biol., 38, 101-109.
47) Coote, JG. 2002. Environmental sensing mechanism in Bordetella. Adv. Microb. Phisiol.44:141-181.
48) COUNCIL DIRECTIVE 2001/18/EC of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC.
49) COUNCIL DIRECTIVE 2001/82/EC of 6 November 2001 on the Community code relating to medicinal products for human use (OJ L 311, 28.11.2001, p. 67.
50) COUNCIL DIRECTIVE 2009/14/EC of 11 March 2009 amending Directive 94/19/EC on deposit-guarantee schemes as regards the coverage level and the payout delay.
51) COUNCIL DIRECTIVE 98/81/EC of 26 October 1998 Overview, General Introduction on Genetically Modified Organisms (GMOs), EU Legislation.
52) Cox, E., Van der Stede, Y., Verdonck, F., Snoeck, V., Van den Broeck, V., Goddeeris, B. 2002. Oral immunisation of pigs with Fimbrial antigens of enterotoxigenic *E. Coli* an interesting model to study mucosal immune mechanism Veterinary Immunology and Immunopathology 87. 287-290

53) Cramer, CL., Weissenborn, DL., Oishi KK., Grabau, E., Bennett, S., Ponce, E., Grabowski GA., Radin, NH. 1996. Bio production of human enzymes in transgenic tobacco. Ann N Y Acad Sci 792, 62-71.

54) Croxen**,** MA., Finlay, BB. 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. Nat Rev Microbiol.; 8(1):26-38.

55) Current Protocols Immunology, Coligen, J.E., et al., eds., John Wiley & Sons, (New York, NY: 1991) sections 8.3.1-8.3.11.

56) Dahlenborg, K., Pound, JD., Borrebaeck, CA., Carlsson, R. 1997. Terminal differentiation in human germinal center B cells *in vitro.* Cell. Immunol. 175, 141-149.

57) Dalloul, RA., Lillehoj, HS. 2005. Recent advances in immunomodulation and vaccination strategies against coccidiosis Avian Dis, 49; 1–8.

58) D'Andrea, S. 2016. Lipid droplet mobilization: the different ways to loosen the purse strings. Biochimie 120, 17–27

59) Daniell, H., Streatfield, SJ., Wycoff, K. 2001b. Medical molecular farming: production of antibodies, biopharmaceuticals, and edible vaccines in plants. Trends Plant Sci 6:219.

60) Davis, M., Kim, S., Hood, L. 1980. DNA sequences mediating class switching in a-immunoglobulins. Science. 209, 1360-1365.

61) Day, CD., Lee, E., Kobayashi, J., Holappa, LD., Albert, H., Ow, DW. 2000 Transgene integration into the same chromosome location can produce alleles that express at a predictable level, or alleles that are differentially silenced. Genes Dev.14:2869–2880.

62) De Greve, H., Wyns, L., Bouckaert, J. 2007. Combining sites of bacterial fimbriae. Curr Opin Struct Biol 17:506–512.

63) De Vetten, N., Wolters, AM., Raemakers. K., Van Der Meer, I., Ter Stege, R., Heeres, E., Heeres, P., Visser, R. 2003. A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. Nat Biotechnol*,* 21, 439-442.

64) De Veylder., M., Montagu, D., Inzé, M. 1997. Herbicide safer inducible gene expression in *Arabidopsis thaliana* Plant Cell Physiol, 38 (5); 568–57.

65) Deruyffelaere, C., Bouchez, I., Morin, H., Guillot, A., Miquel, M., Froissard, M., Chardot, T., D'Andrea, S. 2015. Ubiquitin-mediated proteasomal

degradation of oleosins is involved in oil body mobilization during post-germinative seedling growth in Arabidopsis. Plant Cell Physiol. 56, 1374–1387.

66) Devriendt, B., Goddeeris, BM., Cox, E. 2013. The Fcγ receptor expression profile on porcine dendritic cells depends on the nature of the stimulus. Vet Immunol Immunopathol. 15;152(1-2):43-9.

67) Downey, GP., Granton, JT. 1997. Mechanism of acute lung injury. Curr. Opin Pulm Med. 3(3):234-241.

68) Durandy, A., Honijo, T. 2000. Human genetic defects in class switch recombination (hyper IgM syndrome). C. Opin. Immunol. 13, 543-548.

69) Dus Santos, MJ., Wigdorovitz, A. 2005. Expression of foot and mouth disease virus antigens in transgenic plants Rev Sci Tech; 24(1):175-87.

70) Elowitz, MB., Leibler, S. 2000. A synthetic oscillatory network of transcriptional regulators. Nature. 20;403(6767):335-8.

71) Erdei, A., Kerekes, K., Pecht, I. 1997. Role of C3a and C5a in the activation of mast cells Exp. Clin. Immunogenet. 14(1):16-18.

72) Espinoza, C., Schlechter, R., Herrera, D., Torres, E., Serrano, A., Medina, C., Arce-Johnson, P. 2013. Cisgenesis and Intragenesis: New tools For Improving Crops BiolRes. vol.46 no.4 Santiago.

73) Espinoza-Sánchez, EA., Álvarez-Hernández, MH., Torres-Castillo, JA., Rascón-Cruz, Q., Gutiérrez-Díez, A., Zavala-García, F., Sinagawa-García, SR. 2015. Stable expression and characterization of a fungal pectinase and bacterial peroxidase genes in tobacco chloroplast. Electronic Journal of Biotechnology 18, 161–168.

74) Fairbrother, JM., Nadeau, E., Gyles, CL., 2005. Escherichia coli in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim. Health Res. Rev. 6:17–39.

75) Fernandez-San Millan, A., Mingo-Castel, A., Miller, M., Daniell, H. 2003. A chloroplast transgenic approach to hyper-express and purify Human Serum Albumin, a protein highly susceptible to proteolytic degradation. Plant Biotechnology Journal 1, 71–79.

76) Fiedler, U., Phillips, J., Artsaenko, O., Conrad, U. 1997. Optimization of scFv production in transgenic plants. Immunotechnology, 3, 205-216.

77) Fischer, R., Emans, N., Schuster, F., Hellwig, S., Drossard, J. 1999a. Towards molecular farming in the future: Using plant cell suspension cultures as bioreactors. Biotechnol. Appl. Biochem. 30:113–116.

78) Fischer, R., Neil Emans, N. 2000. Molecular farming of pharmaceutical proteins. Transgenic Research 9: 279–299.

79) Fischer, R., Schillberg, S., Buyel, JF., Twyman, RM. 2013. Commercial aspects of pharmaceutical protein production in plants. *Curr. Pharm. Des.* 19:5471–77

80) Foged**,** C. 2012. siRNA delivery with lipid-based systems: promises and pitfalls. Curr Top Med Chem.;12(2):97-107.

81) Frey, AD., Rimann, M., Bailey, JE., Kallio, PT., Thompson, CJ., Fussengger, M. 2001. Novel pristinamycin-responsive expression systems for plant cells Biotechnol Bioeng, 74 (2); 154–163.

82) Fritz, JH., Rojas, OL., Simard, N., McCarthy, DD., Hapfelmeier, S., Rubino, S., Robertson, SJ., Larijani, M., Gosselin, J., Ivanov, II., Martin, A., Casellas, R., Philpott, DJ., Girardin, SE., McCoy, KD., Macpherson, AJ., Paige, CJ., Gommerman, JL. 2012. Acquisition of a multifunctional IgA1 plasma cell phenotype in the gut. Nature. 481:199-203.

83) Gadaleta, A., Giancaspro, A., Blechl, AE., Blanco, A. 2008. A transgenic durum wheat line that is free of marker genes and expresses 1Dy10. Journal of Cereal Science. 48:439-445.

84) Gao, Y., Zhao, X., Sun, C., Zang, P., Yang, H., Li, R., Zhang, L. 2015 A transgenic ginseng vaccine for bovine viral diarrhea. Virol. J. 12, 73.

85) Garden, AL. 2000. Myogenic and scalp signals evoked by midinspiratory airway occlusion. J Appl Physiol ;89(2):422-9

86) Gelvin, SB. 2003. Improving plant genetic engineering by manipulating the host. Trends Biotechnol. Mar; 21(3):95-98.

87) Georges, F., Saleem, M., Cutler, AJ. 1990. Design and cloning of a synthetic gene for the flounder antifreeze protein and its expression in plant cells. Gene 91(2): 159-165.

88) Gerdtsa, V., Mutwiria, G., Richardsb, J., Van Drunen, S., Van de Hurka, L., Pottera, A. 2013 Carrier molecules for use in veterinary vaccine. Vaccine Vol 31, Issue 4, 11, 596–6.

89) Golovkin, M., Spitsin, S., Andrianov, V., Smirnov, Y., Xiao, Y., Pogrebnyak, N., Markley, K., Brodzik, R., Gleba, Y., Isaacs, SN., Koprowski, H. 2007. Smallpox subunit vaccine produced in Planta confers protection in mice. Proc Natl Acad Sci U S A. 17;104 (16):6864-9.

90) Gonzalez, AE., Gauci, CG., Barber, D.. Gilman, RH., Tsang, VC., Garcia, HH., 2005. Vaccination of pigs to control human neurocysticercosis. Am J Trop Med Hyg, 72, pp. 837–839

91) Gorantala, J., Grover, S., Rahi, A., Chaudhary, P., Rajwanshi, R., Sarin, NB., Bhatnagar, R., 2014. Generation of protective immune response against anthrax by oral immunization with protective antigen plant-based vaccine. Journal of Biotechnology 176, 1–10.

92) Graham, IA. 2008. Seed storage oil mobilization. Annu. Rev. Plant Biol. 59, 115–142.

93) Grubman, MJ., Mason, PW. 2002. Prospects, including time-frames, for improved foot and mouth disease vaccines Rev Sci Tech;21(3):589-600.

94) Gunn, KS., Singh, N., Giambrone, J., Wu, H. 2012. Using transgenic plants as bioreactors to produce edible vaccines. *Journal of Biotech Research*. 2012; 4(1):92–99.

95) Han, KM., Dharmawardhana, P., Arias, R., Ma. C., Busov. V., Strauss, SH. 2011. Gibberellin-associated cisgenes modify growth, stature and wood properties in Populus. Plant Biotechnol J, 9, 162-178.

96) Hansen, G., Shillito, RD., Chilton, MD. 1997. T-strand integration in maize protoplasts after co-delivery of a T-DNA substrate and virulence genes. Proc Natl Acad Sci (USA), 94, 11726-11730

97) Hassan, F., Ren, D., Zhang, W., Merkel, TJ., Gu, XX. 2012. *Moraxella catarrhalis* activates murine macrophages through multiple toll like receptors and has reduced clearance in lungs from TLR4 mutant mice. PLoS ONE; 7(5): e37610.

98) Haverkort, AJ., Struik, PC., Visser, RG., Jacobse, E. 2009. Applied biotechnology to combat late blight in potato caused by Phytophthora infestans. Potato Research, 52, 249-264.

99) Hefferon, H. 2010. Clinical trials fuel the promise of plant-derived vaccines Am J Clin Med, 7 (2010), pp. 30–37

100) Heinrichs, A. 2008. Small RNAs: United in silence. Nature Reviews Molecular Cell Biology. doi: 10.1038/nrm2429

101) Hellens, R., Mullineaux, P., Klee, H. 2000. Technical focus: a guide to Agrobacterium binary Ti vectors. Trends Plant Sci. 5(10):446-51.

102) Henderson, B., Wilson, M. 1996. Microbial / host interactions in health and diseases: who controls the cytokines network? Immunopharmacology 35, 1-21.

103) Hentges, DJ. 1993. The anaerobic microflora of the human body. Clin Infect. Dis. 16, S175-S180.Hofbauer, A., Stoger, E. 2013. Subcellular accumulation and modification of pharmaceutical proteins in different plant tissues. *Curr. Pharm. Des.* 19:5495–502

104) Holme, IB., Dionisio, G., Brinch-Pedersen, H., Wendt, T., Madsen, CK., Vincze, E., Holm, PB. 2012 Cisgenic barley with improved phytase activity. Plant Biotechnol J, 10, 237-247.

105) Honjo, T., Kataoka, T. 1978. Organization of immunoglobulin heavy chain and allelic deletion model. Pro. Nat Acad. Sci. USA75, 2140-2144.

106) Horvath, H., Huang, J., Wong, O., Kohl, E., Okita, T., Kannangara, CG., Wettstein, D., 2000. The production of recombinant proteins in transgenic barley grains. Proc Natl Acad Sci (USA), 97, 1914-1919.

107) Hsieh, K., Huang, AHC. 2004. Endoplasmic reticulum, oleosins, and oils in seeds and tapetum cells. Plant Physiol. 136, 3427–3434.

108) Huang, Z., Elkin, G., Maloney, BJ., Beuhner, N., Arntzen, CJ., Thanavala, Y., Mason, HS. 2005. Virus-like particle expression and assembly in plants: hepatitis B and Norwalk viruses. Vaccine. 7;23(15):1851-8

109) Huang, Z., Phoolcharoen, W., Lai, H., Piensook, K., Cardineau, G., Zeitlin, L., Whaley, KJ., Arntzen, CJ., Mason, HS., Chen, Q. 2010. High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. Biotechnol Bioeng. 1;106(1):9-17.

110) Huang, Z., Phoolcharoen, W., Lai, H., Piensook, K., Cardineau, G., Zeitlin, L., Whaley, KJ., Arntzen, CJ., Mason, HS., Chen, Q. 2010. High-Level Rapid Production of Full-Size Monoclonal Antibodies in Plants by a Single-Vector DNA Replicon System. Biotechnol. Bioeng. 106: 9–17.

111) Huerta, M., De Aluja, AS., Fragoso, G., Toledo, A., Villalobos, N. 2001. Synthetic peptide vaccine against *Taenia solium* pig cysticercosis:

successful vaccination in a controlled field trial in rural Mexico. Vaccine, 20, pp. 262–266

112) Hull, HF., Minor. PD. 2005. When can we stop using oral poliovirus vaccine. J Infect Dis. 15;192(12):2033-5.

113) Imberechts, H., De Greve, H., Schlicker, C., Bouchet, H., Pohl, P. 1992. Characterization of F107 fimbriae of *Escherichia coli* 107/86, which causes edema disease in pigs, and nucleotide sequence of the F107 major fimbrial subunit gene, fedA. Infect Immun 60:1963–1971.

114) Inka Borchers, AM., Gonzalez-Rabade, N., Gray, JC. 2012. Increased accumulation and stability of rotavirus VP6 protein in tobacco chloroplasts following changes to the 5′ untranslated region and the 5′ end of the coding region

115) Ituriaga, G., Jefferson, RA., Bevan, M. 1989. Endoplasmic reticulum targeting and glycosylation of hybrid proteins in transgenic tobacco. Plant Cell, 1, 381-390.

116) Iwasata, T., Shimizu, Honojo, T., Ymagishi, H. 1990. Circular DNA is excised by immunoglobulin class switching recombination, cell 62, 143-149. J Antibiot; 63(8):431-3.

117) Jin, S., Daniell, H. 2014. Expression of gamma-tocopherol methyltransferase in chloroplasts results in massive proliferation of the inner envelope membrane and decreases susceptibility to salt and metal-induced oxidative stresses by reducing reactive oxygen species. Plant Biotechnology Journal 12, 1274–1285. On. Plant Biotechnology Journal 10, 422–434.

118) Jin. XL., Liu, JF., Shen, MX., Dai, Y., Xu, XZ. 2015 Epidemiological investigation of second intermediate hosts of Clonorchissinensis in Pizhou and Xinyi cities of Jiangsu Province. Chinese. 27(6):658-9.

119) Joshi., SG., Schaart, J., Groenwold, R., Jacobsen, E., Schouten, HJ., Krens, FA. 2011. Functional analysis and expression profiling of hcrvf1 and hcrvf2 for development of scab resistant cisgenic and intragenic apples. Plant Mol Biol, 75, 579-591.

120) Jung, J., Choe, J., Li, L., Choi, YS. 2000. Regulation of CD27 expression in the course of germinal center B cell differentiation: the pivotal role of Il-10. Eur. J. Immunol. 30, 2437-2443.

121) Jung, M., Shin, YJ., Kim, J., Cha, SB., Lee, WJ., Shin, MK., Shin, SW. 2014. Induction of immune responses in mice and pigs by oral administration of classical swine fever virus E2 protein expressed in rice calli. Arch. Virol. 159 3219–3230.

122) Kamenarova, K., Abumhadi, N., Gecheff K., Atanassov, A. 2005. Molecular farming in plants: An approach of agricultural biotechnology. Journal of Cell and Molecular Biology. 4: 77-86.

123) Kamthan, A., Chaudhuri, A., Kamthan, M., Datta, A. 2016. Genetically modified (GM) crops: milestones and new advances in crop improvement. Theor Appl Genet; 129(9):1639-55.

124) Karg, SR., Kallio, PT. 2009. The production of biopharmaceuticals in plant systems.

125) Kataoka, T., Kawakami, T., Takahashi, N., Honjo, T. 1980. Rearrangement of immunoglobulin g1 chain gene and mechanism of heavy chain class switching and germinal center formation.

126) Kawai, T., Akira, S. 2005. Pathogen recognition with Toll-like receptors. Curr Opin Immunol; 17(4):338-44.

127) Khandelwal, A., Lakshmi, Sita. Shaila, MS. 2003. Oral immunization of cattle with hemagglutinin protein of rinderpest virus expressed in transgenic peanut induces specific immune responses. Vaccine; 21:3282–3289.

128) Kikkert, M., Hassink, G., Wiertz, E. 2005. The role of the ubiquitination machinery in dislocation and degradation of endoplasmic reticulum proteins Curr Top Microbiol Immunol.; 300:57-93.

129) Kil, DY., Sauber, TE., Jones, DB., Stein**,** HH. 2010. Effect of the form of dietary fat and the concentration of dietary neutral detergent fiber on ileal and total tract endogenous losses and apparent and true digestibility of fat by growing pigs. J Anim Sci.; 88(9):2959-67.

130) Kim, YG., Cha, J., Chandrasegaran, S. 1996. Hybrid restriction enzymes: zinc finger fusions to FokI cleavage domain. Proc Natl Acad Sci USA 93:1156–1160.

131) Kinoshita, K., Tashiro, J., Tomita, S., Lee, C., Honojo, T. 1998. Target specificity of immunoglobulin class switching recombination is not determined by nucleotide sequences os S regions. Immunity 9,849-858.

132) Klein, A., Strecker, G., Lamblin, G., Roussel, P. 2000. Structural analysis of mucin type O-linked oligosaccharides. Method Mol. Bio. 125:191-209.

133) Knowel, MR., Boucher, RC. 2002. Mucus clearance as a primary innate defense mechanism for mammalian airways J. Clin. Invest. 109(5); 571-577.

134) Kohl, TO., Hotzeroth, II., Christensen, ND., Rybicki, EP. 2007. Expression of HPV-11 L1 protein in transgenic Arabidopsis thaliana and *Nicotiana tabacum*. Vaccine 12:7;56.

135) Kolotilin, I., Kaldis, A., Devriendt B., Joensuu, J., Cox, E., Menassa, R. 2004. Production of a subunit vaccine candidate against porcine post-weaning diarrhea in high-biomass transplastomic tobacco. PLoS One; 7: 42405.

136) Kolotilin, I., Kaldis, A., Devriendt, B., Joensuu, J., Cox, E., Menassa, R. 2012. Production of a subunit vaccine candidate against porcine post-weaning diarrhea in high-biomass transplastomic tobacco. PLoS One 7, e42405.

137) Komarova, GI., Babosha, AV. 2010. Polyphase character of the dependence of Brassica napus germ root and hypocotyl growth on zeatin and thidiazuron concentrations with view of applicability to biological life support systems. Aviakosm Ekolog Med.; 44(2):61-5.

138) Koornneef, M., Meinke, D. 2010. The development of *Arabidopsis* as a model plant. Plant J. 61, 909-921.

139) Kopp, EB., Medzhitov, R. 1999. The Toll receptor family and control of innate immunity. Curr. Opin. Immunol. 11(1):13-18.

140) Koya, V., Moayeri, M., Leppla, SH., Daniell, H. 2005. Plant-based vaccine: mice immunized with chloroplast-derived anthrax protective antigen survive anthrax lethal toxin challenge. Infect. Immun. 73, 8266–8274.

141) Kraeling, RR., Webel, SK. 2015. Current strategies for reproductive management of gilts and sows in North America. J Anim Sci Biotechnol. 6(1): 3.

142) Kweon, SM., Wang, B., Rixter, D., Lim, JH., Koga, T., Ishinaga, H. 2006. Synergistic activation of NF-kappa B by non-typeable *H. influenzae* and *S. pneumoniae* is mediated by CK2: IKK-beta-Ikappa-B-alpha, and p38 MAPK. Biochem Biophys Res Commun; 351(2):368–75.

143) Lakshmi, PS., Verma, D., Yang, X., Lloyd, B. Daniell, H. 2013. Low cost tuberculosis vaccine antigens in capsules: expression in chloroplasts, bio-encapsulation, stability and functional evaluation *in vitro* PLoS ONE, 8, e54708

144) Lal, P., Ramachandran, G., Goyal, R., Sharma R. 2007. Edible vaccines: status and future. Indian J Med Microbiol, 25, pp. 93–102.

145) Lamphear, BJ., Jilka, JM., Kesl, L., Welter, M., Howard, JA., Streatfield, SJ. 2004. A corn-based delivery system for animal vaccines: an oral transmissible gastroenteritis virus vaccine boosts lactogenic immunity in swine. Vaccine .22:2420–2424.

146) Langridge, WH., Fitzgerald, KJ., Koncz, C., Schell, JA., SzalayDual, A. 1999. Promoter of *Agrobacterium tumefaciens* mannopine synthase genes is regulated by plant growth hormones Proc Natl Acad Sci U S A, 86 (9); 3219–322.

147) Lau, OS., Sun, SSM. 2009. Plant seeds as bioreactors for recombinant protein production. Biotechnol Adv 27(6):1015–1022.

148) Lauterslager, TG., Florack, EA., Van der Wal, TJ., Molthoff, JW., Langeveld, JPM., Bosch. D., Boersma, WJA., Hilgers, LAT. 2001. Oral immunization of naïve and primed animals with transgenic potato tubers expressing LT-B. Vaccine, 19, 2749-2755.

149) Lee, G., Na, YJ., Yang, BG., Choi, JP., Seo, YB., Hong, CP., Yun, CH. 2015. Oral immunization of hemagglutinin H5 expressed in plant endoplasmic reticulum with adjuvant saponin protects mice against highly pathogenic avian influenza A virus infection. Plant Biotechnol. J. 13, 62–72.

150) Lee, SM., Kang, K., Chung, H., Yoo, SH., Xu, XM., Lee, SB., Cheong, JJ., Daniell, H., Kim, M. 2006. Plastid transformation in the monocotyledonous cereal crop, rice (*Oryza sativa*) and transmission of transgenes to their progeny. Mol Cells 21:401–410.

151) Leelavathi, S., Reddy, VS. 2003. Chloroplast expression of His-tagged GUS-fusions: a general strategy to overproduce and purify foreign proteins using transplastomic plants as bioreactors. Molecular Breeding 11, 49–58.

152) Lehrer, RI., Ganz, T., Selsted, ME. 1991. Defensins endogenous antibiotic peptides of animal cells. Cell 64 (2):229-230.

153) Lentz, EM., Segretin, ME., Morgenfeld, MM., Wirth, SA., Santos, MJD., Mozgovoj, MV., Wigdorovitz, A. 2010. High expression level of a foot and mouth disease virus epitope in tobacco transplastomic plants. Planta, 231, 387–395.

154) Lim, JH., Ha, U., Sakai, A., Woo, CH., Kweon, SM., Xu, H. 2008. *Streptococcus pneumoniae* synergizes with non-typeable *Haemophilus influenzae* to induce inflammation via upregulating TLR2. BMC Immunol; 9:40.

155) Lin, CL., Chen, TF., Chiu, MJ., Way, TD., Lin, JK. 2009. Epigallocatechin gallate (EGCG) suppresses beta-amyloid-induced neurotoxicity through inhibiting c-Abl/FE65 nuclear translocation and GSK3 beta activation. Neurobiol Aging.;30(1):81-92.

156) Lindhout, E., Koopman, G., Pals, ST., De Groot, C. 1997. Triple check for antigen specificity of B cells during germinal center reactions. Immunol Today18:573-577

157) Lindmark, R. 1983. Binding of immunoglobulins to Protein A and immunoglobulin levels in mammalian sera. J. Immunol. Methods, 62(1), 1-13

158) Liu, XY. Rocha-Sosa M., Hummel, JJ., Willmitzer, L., Fromme, WB. 1991. vA. Detail study of the regulation and evolution of the two classes of patatin genes in *Solanum tuberosum* L Plant Mol Biol, 17 (6);1139–1154.

159) Liu, Y., and Arpin, C. (1997). Germinal center development. Immunol. Rev., 156, 11-126.

160) Liu, Y., Arpin, C., de Bouteiller, O., Guret, C., Durand, I., and Banchereau, J. 1995. Memory B cells from human tonsils colonize mucosal ephitelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2.Immunity2, 239-248.

161) Ma, J., Drake, KC., Christou, P. 2003. The production of recombinant pharmaceutical proteins in plants. Nat. Rev. Genet. 4, 794-805.

162) Madec, FN., Bridoux, S., Bounaix, R., Cariolet, I., Duval, Y., Iflah, D., Hampson, A., Jestin. 2000. Experimental models of porcine post-weaning colibacillosis and their relationship to postweaning diarrhea and digestive disorders as encountered in the field. Veterinary Microbiology 72: 295–310.

163) Magnuson, NS., Linzmaier, PM., Reeves, R., An, G., HayGlass, K. Lee, JM. 1998. Secretion of biologically active human interleukin-and interleukin-

4 from genetically modified tobacco cells in suspension culture. Prot. Expr. Purif. 13: 45–52.

164) Majewska, M., Szczepanik, M. 2006. The role of Toll-like receptors (TLR) in innate and adaptive immune responses and their function in immune response regulation. Postepy Hig Med Dosw (Online); 60:52–63.

165) Majumdar, R., Shao, L., Minocha, R., Long, S., Minocha, SC. 2013. Ornithine: the over looked molecule in regulation of polyamine metabolism. Plant Cell Physiology. 54:990–1004.

166) Mandell, CA. 1995. Community acquired pneumonia: etiology epidemiology, and treatment. Chest 108 (2. suppl); 35S-42S.

167) Manis, J., Gu, Y., Lansford, R., Sonoda, E., Ferrini, R., Devidson, L., Rajewsy, K., Alt, F. 1998. Ku 70 is required for late B cells development and immunoglobulin heavy chain switching. J. Exp. Med. 1872081-2089.

168) Mardanova, ES., Kotlyarov, RY., Kuprianov, VV., Stepanova, LA., Tsybalova, LM., Lomonosoff, GP., Ravin, NV. 2015. Rapid high-yield expression of a candidate influenza vaccine based on the ectodomain of M2 protein linked to flagellin in plants using viral vectors. BMC Biotechnol. 29;15:42.

169) Marillonnet, S., Thoeringer, C., Kandzia, R., Klimyuk, V., Gleba, Y. 2005. Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants. Nat Biotechnol;23(6):718-23.

170) Mason, CM., Caufield, CR., Kishinevsky, L. 2010. Sweeping changes to New York's powers of attorney. Care Manag J. ;11(4):220-6.

171) Mason, HS., Arntzen, CJ. 1995. Transgenic plants as vaccine production systems. TIBTECH, 13, 388-392.

172) Mason, HS., Haq, TA., Clements. JD., Arntzen, CJ. 1998. Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic LT B gene. Vaccine, 16, 1336-1343.

173) Matsumoto, K., Nagatake T. 1990. Drug therapy of intractable methicillin-resistant staphylococcal infections]. Nihon Naika Gakkai Zasshi. 10;79(12):1663-8.

174) McClements DJ, Li, Y. 2010. Review of *in vitro* digestion models for rapid screening of emulsion-based systems. Food Funct. 1, 32–59.

175) McGarvey, PB., Hammond, J., Dienelt, MM. 1995. Expression of the rabies virus glycoprotein in transgenic tomatoes. *Nature Biotechnology*; 13(13):1484–1487. doi: 10.1038/nbt1295-1484.

176) Medzhitov, R., Janeway, CA. Jr. 2002. Decoding the patterns of self and non self by the innate immune system. Science; 296(5566):298–300.

177) Medzhitov, R., Preston-Hurlburt, P., Janeway, CA. Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. nature 388(6640):394-397.

178) Mehra, UR., Verma, AK., Dass, RS., Sharma, RL., Yadav, SC. 1999. Effects of *Fasciola gigantica* infection on growth and nutrient utilization of buffalo calves. Vet Res; 145:699–70.

179) Moehn, S., Levesque, CL., Ball, RO. 2013. Protein intake but not feed intake affects dietary energy for finishing pigs..J Anim Physiol Anim Nutr (Berl).97(1):197-204.

180) Molina, A., Hervás-Stubbs, S., Daniell, H., Mingo-Castel, AM., Veramendi, J. 2004 High-yield expression of a viral peptide animal vaccine in transgenic tobacco chloroplasts. Plant Biotechnol. J. 2, 141–153.

181) Mollenkvist, A., Nordstrom, T., Hallden, C., Christensen, JJ., Forsgren, A., Riesbeck, K. 2003. The *Moraxella catarrhalis* IgD-binding protein MID is highly conserved and regulated by a mechanism corresponding to phase variation. J. Bacteriol.185,2285–229.

182) Monsanto-Cargill venture takes root. Illinois Agrinews. September 15, 2006.

183) Moonens, K., De Kerpel, M., Coddens, A., Cox, E., Pardon, E. 2014. Nanobody Mediated Inhibition of Attachment of F18 Fimbriae Expressing Escherichia coli PLoS ONE 9(12) e114691.

184) Morgenfeld M Lentz E Segretin ME Alfano EF Bravo-Almonacid F. 2014. Translational fusion and redirection to thylakoid lumen as strategies to enhance accumulation of human papillomavirus E7 antigen in tobacco chloroplasts. Molecular Biotechnology 56, 1021–1031.

185) Müller, B., Dürr, S., Alonso, S., Hattendorf, J., Laisse, C., Parsons, SD., Van Helden, PD. 2013. Zoonotic Mycobacterium bovis-induced tuberculosis in humans. Emerg. Infect. Dis. 19, 899–908.

186) Murphy, TF., Faden, H., Bakaletz LO. 2009. Non-typeable *Haemophilus influenza* as a pathogen in children. Pediatr Infect Dis J. 28:43–8.

187) Musiychuk, K., Stephenson, N., Bi, H., Farrance, CE., Orozovic, G., Brodelius, M., Brodelius, P., Horsey, A., Ugulava, N., Shamloul, AM., Mett, V., Rabindran, S., Streatfield, SJ., Yusibov, 2007. A launch vector for the production of vaccine antigens in plants. Influenza Other Respir Viruses.;1(1):19-25.

188) Nadai, M., Bally, J., Vitel, M., Job, C., Tissot, G., Botterman, J., Dubald, M. 2009. High-level expression of active human alpha1-antitrypsin in transgenic tobacco chloroplasts. Transgenic Research 18, 173–183.

189) Nagy, B., Fekete, PZ. 2005 Enterotoxigenic *Escherichia coli* in veterinary medicine. Int J Med Microbiol 295:443–454.

190) Nakamura-Uchiyama, F., Tokunaga, Y., Suzuki, A., Akao, N., Hiromatsu, F., Hitomi, S., 2006. A case of *Ascaris suum* visceral larva migrans diagnosed by using *A. suum* larval excretory-secretory (ES) antigen Scand J Infect Dis, 38; 221–224.

191) Nelson, G., Marconi, P., Periolo, O., La Torre, J., Alvarez, MA. 2012 Immunocompetent truncated E2 glycoprotein of bovine viral diarrhea virus (BVDV) expressed in *Nicotiana tabacum* plants: a candidate antigen for new generation of veterinary vaccines. Vaccine, 30, 4499–4504.

192) Newell, KW. 2000. Preparing for the health needs for the year. N Z Med J. 22; 87(606):140-3.

193) Nielsen, AA., Segall-Shapiro, TH., Voigt, CA. 2013 Advances in genetic circuit design: novel biochemistries, deep part mining, and precision gene expression**.** Curr Opin Chem Biol.; 17(6):878-92.

194) Nielsen, K. 2003. Transgenic organisms—time for conceptual diversification? Nature Biotechnology 21, 227 – 228.

195) Novak, N., Koch, S., Allam, JP., Bieber, T. 2010. Dendritic cells: bridging innate and adaptive immunity in atopic dermatitis. J Allergy Clin Immunol; 125(1):50–9.

196) Obembe, OO., Popoola, O., Leelavathi, S., Reddy, SV. 2011. Advances in plant molecular farming. Biotechnol. Adv. 29, 210-222.

197) O'Brien, AD., Holmes, RK. 1987. Shiga and Shiga-like toxins. Microbiol Rev.; 51(2):206-20.

198) Odell, JT., Nagy, F., Chua, NH. 1985. Identification. of DNA sequence required for activity of the cauliflower mosaic virus 35S promoter. Nature, 313 (6005) ; 810–812.

199) Ohya, K., Matsumura, T., Itchoda, N., Ohashi, K., Onuma, M., Sugimoto, C. 2005. Ability of orally administered IFN-α-containing transgenic potato extracts to inhibit Listeria monocytogenes infection. J. Interferon Cytokine Res. 25, 459–466.

200) Ortigosa, SM., Fernández-San Millán, A., Veramendi, J. 2010. Stable production of peptide antigens in transgenic tobacco chloroplasts by fusion to the p53 tetramerisation domain. Transgenic Res. 19, 703–709.

201) Owm DW. 2005. Transgene management via multiple site-specific recombination systems. In Vitro Cellular and Developmental Biology—Plant. 41:213-219.

202) Park, Y., Cheong, H. 2002. Expression and production of recombinant human interleukin-2 in potato plants. Protein Expression Purification, 25,160–165.

203) Parmenter, DL., Boothe, JG., van Rooijen, GJ., Yeung, EC., Moloney, MM. 2003 Production of biologically active hirudin in plant seeds using oleosin partitioning. Plant Mol Biol.;29(6):1167-80.

204) Pasare, C., Medzhitov, R. 2005. Control of B-cell responses by Toll-like receptors. Nature; 438(7066):364–8.

205) Pasquevich, KA., Ibañez, AE., Coria, LM., García Samartino, C., Estein, SM., Zwerdling, A., Barrionuevo, P., Oliveira, FS., Seither, C., Warzecha, H., Oliveira, SC., Giambartolomei, GH., Cassataro, J. 2011. An oral vaccine based on U-Omp19 induces protection against B. abortus mucosal challenge by inducing an adaptive IL-17 immune response in mice. PLoS One.; 14;6(1).

206) Paul, M., Ma, JK. 2010. Plant-made immunogens and effective delivery strategies. Expert Rev Vaccin 9: 821–33.

207) Paul, MJ., The, AY., Twyman, RM., Ma, JK. 2013. Target product selection—where can molecular pharming make the difference? *Curr. Pharm. Des.* 19:5478–85.

208) Paul, MJ., The, AY., Twyman, RM., Ma, JK. 2013. Target product selection—where can molecular pharming make the difference?. Current Pharmaceutical Design. 19:5478–85.

209) Pearay, L. Ogra,* Faden, H., Welliver, R. 2004. Vaccination Strategies for Mucosal Immune Responses. Clin Microbiol Rev. 14(2): 430–445.

210) Pelham, HRB. 1990. The retention signal for soluble proteins of the endoplasmic reticulum. TIBS, 15, 483-486.

211) Pelosi, A., Piedrafita, D., De Guzman, G., Shepherd, R., Hamill, JD., Meeusen, E., Walmsley, AM. 2012. The effect of plant tissue and vaccine formulation on the oral immunogenicity of a model plant-made antigen in sheep. PLoS One.;7(12).

212) Perez Filgueira, DM., Zamorano, PI., Dominguez, MG., Taboga, O., Del Medico, Zajac, MP., Puntel, M., Romera, SA., Morris, TJ., Borca, MV., Sadir, AM. 2003. Bovine herpes virus gD protein produced in plants using a recombinant tobacco mosaic virus (TMV) vector possesses authentic antigenicity. Vaccine;21: 4201–4209.

213) Permyakova, NV., Zagorskaya, AA., Belavin, PA., Uvarova, EA., Nosareva, OV., Nesterov, AE., Novikovskaya, AA. 2015. Transgenic carrot expressing fusion protein comprising *M. tuberculosis* antigens induces immune response in mice. BioMed Res. Int. 2015, 11pp. doi:10.1155/2015/417565

214) Perrin P, Jacob Y, Desmézières E, Tordo N. 2000. DNA-based immunization against rabies and rabies-related viruses: towards multivalent vaccines Dev Biol (Basel).;104:151-7.

215) Petersen, K., Bock, R. 2011. High-level expression of a suite of thermostable cell wall-degrading enzymes from the chloroplast genome. Plant Molecular Biology 76, 311–321.

216) Phan, G, Remaut, H., Wang, T., Allen, WJ., Pirker, KF. 2011. Crystal structure of the FimD usher bound to its cognate FimC-FimH substrate. Nature 474:49–53.

217) Philip, R., Darnowski, DW., Maughan, PJ., Vodkin, LO. 2001. Processing and localization of bovine beta-casein expressed in transgenic soybean seeds under control of a soybean lectin expression cassette. Plant Sci.;161(2):323-335

218) Phoolcharoen, W., Bhoo, S., Lai, H., Ma, J., Arntzen, C.J., Chen, Q., Mason, H.S. 2011. Expression of an immunogenic Ebola immune complex in *Nicotiana benthamiana*. Plant Biotechnol. J.; 9:807–816. Plant Biotechnol J.; 13(8):1013-6.

219) Pluske, JR., Fenton, TW., Lorschy, ML., Pettigrew, JE., Sower, AF., Aherne, FX. 1997. A modification to the isotope-dilution technique for estimating milk intake of pigs using pig serum J Anim Sci.; 75(5):1279-83.

220) Potter, A., Gerdts, V., Litte-van den Hurk, S. 2008. Veterinary vaccines: alternatives to antibiotics? Anim Health Res Rev. 9:187–199.

221) Puigbò, P., Guzmán, E., Romeu, A., Garcia-Vallvé, S. 2007. OPTIMIZER: a web server for optimizing the codon usage of DNA sequences. Nucleic Acids Res., 35, W126. 50.

222) Rabbitts, T., Forster, A., Dunnick, W., Bentley, D. 1980. The role of gene deletion in in the immunoglobulin heavy chIN SWITCH. Nature. 283. 351-356.

223) Rigano, MM., Scotti, N., Cardi, T. 2012. Unsolved problems in plastid transformation. Bioengineered. 3(6):329-333.

224) Rippinger, P., Bertschinger, HU., Imberechts, H., Nagy, B., Sorg, I., Stamm, M., Wild P, Wittig W Designations F18ab and F18ac for the related fimbrial types F107, 2134P and 8813 of Escherichia coli isolated from porcine postweaning diarrhoea and from oedema disease. Vet Microbiol.;45(4):281-95.

225) Rolink, A., Melchers, F., Andersson, J. 1996. The SCID but not the RAG-2 gene product is required for S mu-S epsilon heavy chain class switching. Immunity 5, 319-330.

226) Rommens, CM., Bougri, O., Yan, H., Humara, JM., Owen, J., Swords, K., Ye, J. 2005. Plant-derived transfer DNAs. Plant Physiol, 139, 1338-1349.

227) Rommens, CM., Haring, MAò, Swords Kò., Davies, HV., Belknap, WR. 2007. The intragenic approach as a new extension to traditional plant breeding. *Trends Plant Sci,* 12, 397-403.

228) Rommens, CM., Richael, CM., Yan, H., Navarre, DA., Ye, J., Krucker, M., Swords, K. 2008. Engineered native pathways for high kaempferol and caffeoylquinate production in potato. Plant Biotechnol J.;6(9):870-86.

229) Rommens, CM., Ye, J., Richael. C., Swords, K. 2006. Improving potato storage and processing characteristics through all-native DNA transformation. J Agric Food Chem, 54, 9882-9887.

230) Rossi, L., Dell'Orto, V., Sala, V., Reggi, S., Baldi, A. 2014. Protective effect of oral administration of transgenic tobacco seeds against Vero cytotoxic *Escherichia coli* strain in piglets. Vet Res Comm. 38 (1): 39-49.

231) Rossi, L., Fusi, E., Baldi, G., Fogher, C., Cheli, F., Baldi, A., Dell'Orto, V. 2013. Tobacco seeds by-product as protein source for piglets. Open Journal of Veterinary Medicine 3, 73-78.

232) Rossi, L., Reggi, S., di Giancamillo, A., Domeneghini, C., Pinotti, L., C. Fogher, C., Baldi, A. 2003 a. Oral administration of tobacco seeds expressing Antigenic Proteins in mice Balb-C: A model of oedema disease Italian journal of animal science. Vol 2 N° 1 pp7-9.

233) Rossi, L., Reggi, S., Fogher, C., Vagni, S., Baldi, A. 2011. Evaluation of gastric degradability of antigenic protein expressed in tobacco seeds. IJASs 1:19.

234) Rossi, L., Selmini, G., Cheli, F., Fusi, E., Fogher. C. 2007. Potential use of tobacco seed cake in piglet's diet. Large Animal Review, 13(5): 211-215.

235) Ruan, M., Akkoyunlu, M., Grubb, A., Forsgren, A. 1990. Protein D of *Heamophilus influenza*e. A novel bacterial surface protein with affinity for human IgD. Immunol. 145; 3379-3384.

236) Ruebelt, MC., Lipp, M., Reynolds, TL., Astwood, JD., Engel, KH., Jany, KD. 2006. Application of two dimensional gel electrophoresis to interrogate alterations in the proteome of genetically modified crops. Assessing natural variability. J. Agric. Food Chem. 54, 2162-2168.

237) Ruf, S., Hermann, M., Berger, IJ., Carrer, H., Bock, R. 2001. Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. Nature Biotechnology 19, 870–875.

238) Ruiz, ON., Hussein, HS., Terry, N., Daniell, H. 2003. Phytoremediation of organomercurial compounds via chloroplast genetic engineering. Plant Physiology 132, 1344–1352.

239) Saldaña, S., Guadarrama, FE., Olivera Flores, TDJ.2006. Production of rotavirus-like particles in tomato (*Lycopersicon esculentum* L.) fruit by

expression of capsid proteins VP2 and VP6 and immunological studies. *Viral Immunology*; 19(1):42–53. doi: 10.1089/vim.2006.19.42.

240) Samuelsson, M., Hallstrom, T., Forsgren, A., Riesbeck, R. 2007. Characterization of the IgD binding site of encapsulated *Haemophilus influenzae* serotype b. *J Immunol 178:6316-9.*

241) Sangild, PT., 2003. Uptake of colostral immunoglobulins by the compromised newborn farm animal. Acta Vet Scand Suppl. 98:105–122.

242) Sathish, K., Sriraman, R., Subramanian, BM., Rao, NH., Balaji, N., Narasu ML. 2011. Plant expressed EtMIC2 is an effective immunogen in conferring protection against chicken coccidiosis Vaccine, 29; 9201–9208.

243) Schaart, JG., Krens, FA., Pelgrom, KT., Mendes, O., Rouwendal, GJ. 2004. Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. Plant Biotechnol J.;2(3):233-40.

244) Schillberg, S., Fischer, R., Emans, N. 2003. Molecular farming' of antibodies in plants. Naturwissenschaften; 90(4):145-55.

245) Schillberg, S., Twyman, RM., Fischer, R. 2005. Opportunities for recombinant antigen and antibody expression in transgenic plants--technology assessment. Vaccine. 7; 23(15).:1764-9.

246) Schnare, M., Barton, GM., Holt, AC., Takeda, K., Akira, S., Medzhitov, R. 2001. Toll-like receptors control activation of adaptive immune responses. Nat Immunol; 2 (10):947–50.

247) Schouten, A., Roosien, J., Van Engelen, FA., De Jong, GA., Borst, M., Vrenssen, AWM., Zilverentant, JF., Bosch, D., Stiekema, WJ., Gommers. FJ., Schots, A., Bakker, J 1996. The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. Plant Mol. Biol., 30, 781–793.

248) Schouten, HJ., Krens, FA. Jacobsen, E. 2006a. Cisgenic plants are similar to traditionally bred plants: international regulations for genetically modified organisms should be altered to exempt cisgenesis. EMBO Rep, 7, 750-753.

249) Sciutto, E., Fragoso, G., Fleury, A., Laclette, JP., Sotelo, AJ., Aluja. A. 2000. *Taenia solium* disease in humans and pigs: an ancient parasitosis disease

rooted in developing countries and emerging as a major health problem of global dimensions Microbes Infect, 15; 1875–1890.

250) Sciutto, E., Morales, J., Martinez, J., Toledo, A., Villalobos, M.N. 2007 Further evaluation of the synthetic peptide vaccine S3Pvac against *Taenia solium* cysticercosis in pigs in an endemic town of Mexico Parasitology, 134, pp. 129–133.

251) Sears, CL., Kaper, JB., 1996. Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. Microbiol Rev 60:167–215.

252) Sharm, AK., Sharma, MK. 2009. Plants as bioreactors: recent developments and emerging a opportunities. Biotechnol Adv 27:811–32.

253) Shaw, CH., Carter GH., Watson MD., Shaw, CH. 1984. A functional map of the nopaline synthase promoter Nucleic Acids Res, 12 (20); 7831–7846 R.G.

254) Shinmyo, A., Kato, K. 2010. Molecular farming: production of drugs and vaccines in higher plants.

255) Shirley, MH., Smith, AL., Tomley, FL. 2005. The biology of avian *Eimeri*a with an emphasis on their control by vaccination. Adv Parasitol, 60; 285–330.

256) Siloto, RMP., Findlay, K,. Lopez-Villalobos, A., Yeung, EC., Nykiforuk, CL., Moloney, MM. 2006. The accumulation of oleosins determines the size of seed oilbodies in Arabidopsis. Plant Cell Online 18, 1961–1974.

257) Sirskyi, D., Kumar, A., Azizi,A. 2016. Mechanisms Underlying the Immune Response Generated by an Oral Vibrio cholerae Vaccine. Int.J Mol Sci. 2;17(7). pii: E1062

258) Sjöquist, J. 1972. Protein A isolated from *Staphylococcus aureus* after digestion with lysostaphin. Eur. J. Biochem., 29, 572–578.

259) Smeds, A., Hemmann, K., Jakava-Viljanen, M., Pelkonen, S., Imberechts, H. 2001. Characterization of the adhesin of *Escherichia coli* F18 fimbriae. Infect Immun 69:7941–7945.

260) Soria-Guerra**,** RE., Rosales-Mendoza, S., Moreno-Fierros, L., López-Revilla, R., Alpuche-Solís, AG. 2010. Oral immunogenicity of tomato-derived sDPT polypeptide containing *Corynebacterium diphtheriae, Bordetella*

pertussis and *Clostridium tetani* exotoxin epitopes. Plant Cell Rep.; 30(3):417-24.

261) Spök, A., Karner, S. 2008. Plant Molecular Farming, Opportunities and Challenges. Office for Official Publications of the European Communities; Kopstal, Luxembourg: JRC Technical Report EUR 23383 EN.

262) Srivastava, V., Anderson, OD., Ow, DW. 1999. Single-copy transgenic wheat generated through the resolution of complex integration patterns. Proc Natl Acad Sci USA 96:11117–11121.

263) Srivastava, V., Ariza-Nieto, M., Wilson, AJ. 2004. *Cre*-mediated site-specific gene integration for consistent transgene expression in rice. Plant Biotechnol J.; 2:169–179.

264) Stahl, R., Horvath, H., Van Fleet, J., Voetz, M., von Wettstein, D., Wolf, N. 2002. T-DNA integration into the barley genome from single and double cassette vectors. Proc. Natl. Acad. Sci. U. S. A. 99, 2146-2151.

265) Staub, JM., Garcia, B., Graves, J. 2000. High-yield production of a human therapeutic protein in tobacco chloroplasts. Nature Biotechnology 18, 333–338.

266) Stoger, E., Fischer, R., Moloney, M., Ma, JK. 2014. Plant molecular pharming for the treatment of chronic and infectious diseases. Annu Rev Plant Biol.; 65:743-68.

267) Stöger, E., Vaquero, C., Torres, E., Sack, M., Nicholson, L., Drossard, J., Williams, S., Keen, D., Perrin, Y., and Christou, P. 2000. Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. Plant Mol. Biol. 42, 583-590.

268) Streatfeld, SJ., Meyer, JM., Barker, DK., Brooks, C-, Lamphear, BJ., Woodard, SL., Beifuss, KK., Vicuna, DV., Massey, LA., Horn, ME., Delaney, DE., Nikolov, ZL, Hood, EE., Jilka, JM., Howard, JA. 2002. Development of an edible subunit vaccine in corn against enterotoxigenic strains of *Escherichia coli.* In Vitro Cell. Dev. Biol.-Plant, 38, 11-17.

269) Streatfield, SJ. 2005 Delivery of plant-derived vaccines. Expert Opin Drug Deliv, 2 pp. 719–728.

270) Streatfield, SJ. 2006. Mucosal immunization using recombinant plant-based oral vaccines. Methods. ;38(2):150-7.

271) Su, CC., Chu, JY. 2015. Taiwan Medical Association. Japan Med Assoc J. 1;58(4):285-8.

272) Sullivan, ML., Green, PJ. 1993. Post-transcriptional regulation of nuclear-encoded genes in higher plants: the roles of mRNA stability and translation. Plant Mol Biol. 23(6):1091-104.

273) Sutton, D., Havstad, PK., Kemp, JD. 1992. Synthetic cryIIIA gene from *Bacillus thuringiensis* improved for high expression in plants. Trans. Res. 1: 228-236.

274) Svab, Z., Hajdukiewicz, P., Maliga, P. 1990a. Stable transformation of plastids in higher plants. Proceedings of the National Academy of Sciences of the United States of America 87, 8526–8530.

275) Szarka, S., Van Rooijen, G. Moloney, M. 2006. Methods for the production of multimeric immunoglobulins, and related compositions. United States Patent 7. 098,383.

276) Tackaberry, ES·, Dudani, AK., Prior, F., Tocchi, M., Sardana, R., Altosaar, I., Ganz,PR. !999. Development of biopharmaceuticals in plant expression systems: cloning, expression and immunological reactivity of human cytomegalovirus glycoprotein B (UL55) in seeds of transgenic tobacco. Vaccine. 6;17(23-24):3020-9.

277) Tacket, CO., Mason, HS., 1999. A review of oral vaccination with transgenic vegetables Microbes Infect, 1, pp. 777–783.

278) Tacket, CO., Mason, HS., Losonsky, G., Clements, JD., Levine, MM., Arntzen, CJ. 1998. Immunogenicity in humans of a recombinant bacterial-antigen delivered in transgenic potato. Nat Med, 4, 607–609.

279) Tacket, CO., Mason, HS., Losonsky, G., Estes, MK., Levine, MM., Arntzen, CJ. 2000. Human immune responses to a novel norwalk virus vaccine delivered in transgenic potatoes. J Infect Dis., 182, 302–305.

280) Tacket, CO., Pasetti, MF., Edelman, R., Howard, JA., Streatfield, S. 2004. Immunogenicity of recombinant LT-B delivered orally to humans in transgenic corn. Vaccine, 22, 4385–4389.

281) Tannock, GW. 1995. Normal microflora: an introduction of microbiome inhabiting the human body, 1-115. London: Chapman and Hall.

282) Tissot, G., Canard, H., Nadai, M., Martone, A., Botterman, J., Dubald, M. 2008. Translocation of aprotinin, a therapeutic protease inhibitor, into the thylakoid lumen of genetically engineered tobacco chloroplasts. Plant Biotechnology Journal 6, 309–320.

283) Toledo, A., Fragoso, G., Rosas, G., Hernandez, M., Gevorkian, G., Lopez-Casillas, F. 2001. Two epitopes shared by *Taenia crassiceps* and *Taenia solium* confer protection against murine *T. crassiceps* cysticercosis along with a prominent T1 response Infect Immun, 69, pp. 1766–1773.

284) Toledo, A., Larralde, C., Fragoso, G., Gevorkian, G., Manoutcharian, K., Hernandez, M. 1999. Towards a *Taenia solium* cysticercosis vaccine: an epitope shared by *Taenia crassiceps* and *Taenia solium* protects mice against experimental cysticercosis Infect Immun, 67, pp. 2522–2530.

285) Tong, HH., Blue, LE., James, MA., DeMaria, TF. 2000. Evaluation of the virulence of a *Streptococcus pneumoniae* neuramidase-deficent mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. Infect. Immu. 68(2):921-924.

286) Topp, E., Irwin, R., McAllister. T., Lessard, M., Joensuu, J., Kolotilin, U., Stoger, E., Warzecha, H., Hall, C., McLean, D., Cox, E., Davrient, B., Potter, A., Depicker, A., Virdi, V., Holbrook, L., Doshi, K., Dussalult, M., Yoo, H. 2016. The case for plant-made veterinary immunotherapeutic. Biotechnology advances 34; 597-604.

287) Torrent, M., Llop-Tous, I., Ludevid, MD. 2009b. Protein body induction: a new tool to produce and recover recombinant proteins in plants. Methods Mol Biol 483:193–208.

288) Tort, PJ., Brindley, D., Knox, KH., Wolfe, JP. 1999. Dalton Proteinases and associated genes of parasitic helminth Adv Parasitol, 43 ; 161–166.

289) Tregoning, J., Maliga, P., Dougan, G., Nixon, PJ. 2004. New advances in the production of edible plant vaccines: chloroplast expression of a tetanus vaccine antigen, TetC. Phytochemistry.;65(8):989-94.

290) Tsuji, NK., Suzuki, H., Kasuga-aoki, T., Isobe, T., Arakawa, Y. 2003. Mice intranasally immunized with a recombinant 16-kilodalton antigen from roundworm Ascaris parasites are protected against larval migration of *Ascaris suum.* Infect Immun, 71; 5314–5323.

291) Van den Broeck, W., Cox, E., Goddeeris, BM. 1999. Receptor-dependent 681 immune responses in pigs after oral immunization with F4 fimbriae. Infect Immun 67: 520-6.

292) Van Droogenbroeck, B., Cao, J., Stadlmann, J., Altmann, F., Colanesi, S., Hillmer, S., Robinson, D.G., Van Lerberge, E., Terryn, N., and Van Montagu, M. 2007. Aberrant localization and underglycosylation of highly accumulating single-chain Fv-Fc antibodies in transgenic Arabidopsis seeds. Proceedings of the National Academy of Sciences 104, 1430-1435.

293) Vanblaere, T., Szankowski, I., Schaart, J., Schouten, H., Flachowsky, H., Broggini, GAL., Gessler, C. 2011. The development of a cisgenic apple plant. Journal of Biotechnology. 154:304–311.

294) Verdonck, F., Cox, E., van Gog, K., Van der Stede, Y., Duchateau, L., Deprez, P., Goddeeris, BM. 2002. Different kinetic of antibody responses following infection of newly weaned pigs with an F4 enterotoxigenic *Escherichia coli* strain or an F18 verotoxigenic *Escherichia coli* strain. Vaccine 20:2995–3004.

295) Verma, D., Moghimi, B., LoDuca, PA., Singh, HD., Hoffman, BE., Herzog, RW., Daniell, H. 2010b. Oral delivery of bioencapsulated coagulation factor IX prevents inhibitor formation and fatal anaphylaxis in hemophilia B mice. Proceedings of the National Academy of Sciences of the United States of America 107, 7101–7106.

296) Villalobos, A., Ness, JE., Gustafsson, C., Minshull, J., Govindarajan, S. 2006. Gene Designer: a synthetic biology tool for constructing artificial DNA segments. BMC Bioinform., 7, 285.

297) Virdi, V., Depicker, A. 2013. Role of plant expression systems in antibody production for passive immunization. Int J Dev Biol; 57(6-8):587-93.

298) Visser, A., Stolte, E., Jacobsen, R. 1991. Expression of a chimeric granule-bound starch synthase-GUS gene in transgenic potato plants. Plant Mol Biol, 17 (4); 691–699.

299) Vitovski, S., Dunkin, KT., Howard, AJ., Sayers, JR. 2002. Nontypeable *Haemophilus influenzae* in carriage and disease: a difference in IgA1 protease activity levels. JAMA; 287:1699-705.

300) Vuononvirta, J., Toivonen, L., Grondahl-Yli-Hannuksela, K., Barkoff, AM., Lindholm, L., Mertsola, J. 2007 Nasopharyngeal bacterial colonization and gene polymorphisms of mannose-binding lectin and Toll-like receptors 2 and 4 in infants. PLoS ONE; 6(10): e26198.

301) Walmsley, AM. and Arntzen, CJ. 2000. Plants for delivery of edible vaccines. Current Opinion in Biotechnology. 11:126–129.

302) Walmsley, AM., Arntzen, CJ. 2003. Plant cell factories and mucosal vaccines Curr Opin Biotechnol. (2):145-50.

303) Wang, X., Moser, C., Loubutin, JP., Wilson, JM. 2002. Toll Like receptor 4 mediates innate immune responses to *Haemophilus influenzae* infection in mouse lung. J. Immunol. 168(2):810-815.

304) Warzecha, H., Mason, HS. 2003. Benefits and risks of antibody and vaccine production in transgenic plants. J Plant Physio;160(7):755-64.

305) Warzechaa, H., Mason, HS. 2003. Benefits and risks of antibody and vaccine production in transgenic plants. Journal Plant Physiology. 160:755–764.

306) Waters, M., Pyke, K., 2005. Plastid development and differentiation. In: Moller SG, ed. Annual reviews: Plastids. Oxford: Blackwell, 30–59.

307) Weeks, JT., Ye, J., Rommens, CM. 2008. Development of an in planta method for transformation of alfalfa (*Medicago sativa*). Transgenic Res, 17, 587-597.

308) Whitman, GJ., Hart, JC., Crestanello, JA., Spooner, TH. 1999. Uniform safety of beating heart surgery using the octopus tissue stabilization system. J Card Surg; 14(5):323.

309) Wilson, R., Read, R., Thomas, M., Rutman, A., Harrison, K., Lund, V., Cole, P. 1991. Effect of *Bordetella pertussis* infection of human respiratory epithelium in vivo and *in-vitro*. Infect. Immu. 59(1):337-345.

310) Xie, Y., Liu, Y., Meng M., Chen, L., Zhu, Z. 2004. Isolation and identification of a super strong plant promoter from cotton leaf curl Multan virus Plant Mol Biol, 53 (1); 1–14.

311) Xue, GP. 2003. The DNA binding activity of an AP2 transcriptional activator HvCBF2 involved in regulation of low temperature responsive genes in barley is modulated by temperature. Plant J. 33, 373-383.

312) Yamasaki, H., Mineki, R., Murayama, K., Ito, A., Aoki, T. 2002. Characterization and expression of the *Fasciola gigantica* cathepsin L gene Int J Parasitol, 32. 1031–1042.

313) Yang, L., Wang, H., Liu, J., Li, L., Fan, Y., Wang, X., Songa, Y., Suna, S., Wang, L., Zhua, X., Wang, X. 2008. A simple and effective system for foreign gene expression in plants via root absorption of agrobacterial suspension. Journal of Biotechnology. 134:320–324.

314) Yang, Y., Li, R., Qi, M. 2000. In vivo analysis of plant promoters and transcription factors by agro infiltration of tobacco leaves. The Plant Journal. 22(6):543-551.

315) Yian, Y., Yunqi, W., Dickey, A., Yueju, W. 2015. Plants as Factories for Human Pharmaceuticals: Applications and Challenges Int J Mol Sci. 16(12): 28549–28565. doi: 10.3390/ijms161226122.

316) Zhang, J., Ruf, S., Hasse, C., Childs, L., Scharff, LB., Bock, R. 2012. Identification of cis-elements conferring high levels of gene expression in non-green plastids. The Plant Journal. 72:115–128.

317) Zhang, JY., Marris, C., Rose, N. 2011. The Transnational Governance of Synthetic Biology Scientific uncertainty, cross-borderness and the 'art' of governance. BIOS Working Paper, BIOS, London School of Economics and Political Science, London. 4:2-36.

318) Zhang, W., Bielaszewska, LM.,. Kuczius T., Karch**,** H. 2002. Identification, characterization, and distribution of a Shiga toxin 1-gene variant (*stx*$_{1c}$) in *Escherichia coli* strains isolated from humans. J. Clin. Microbiol. 40**:**1441-1446.

319) Zhang, XH., Brotherton, JE., Widholm, JM., Portis, AR. 2001. Targeting a nuclear anthranilate synthase alpha-subunit gene to the tobacco plastid genome results in enhanced tryptophan biosynthesis: return of a gene to its pre-endosymbiotic origin. Plant Physiology 127, 131–141.

320) Zhou, JY., Wu, JX., Cheng, LQ. 2003. Expression of immunogenic S1 glycoprotein of infectious bronchitis virus in transgenic potatoes. Journal of Virology; 77(16):9090–9093. doi: 10.1128/JVI.77.16.9090-9093.2003.

321) Zimmermann, J., Saalbach, I., Jahn, D., Giersberg, m., Haehnel, S., Wedel, J., Macek, J., Zoufal, K., Glünder, G., Falkenburg, D., Kipriyanov, SM. 2009. Antibody expressing pea seeds as fodder for prevention of gastrointestinal parasitic infections in chickens. BMC Biotechnology. 9(79):1-22.

ACKNOWLEDGEMENTS

This project has been funded with support from the M.I.U.R-Regione Lombardia, ID N° 30210444 (Progetti di Ricerca Industriale e Sviluppo Sperimentale nei Settori strategici di Regione Lombardia e del Ministero dell'Istruzione, dell'Universita' e della Ricerca di cui al Decreto n.° 7128 del 29 luglio 2011) and it was developed in collaboration with Ferraroni S.P.A, Plantechno s.r.l., Department VESPA and the Department DIVET of the University of Milan and the diagnostic section of Brescia of the I.Z.S.L.E.R..

I thankfully acknowledge Prof. Antonella Baldi, the scientific responsible of the project for the University of Milan and of the ethical authorization for the use of animals for a scientific purpose (102/2015 PR Health Ministry), Dott. Luciana Rossi responsible of the procedure on animals. Prof. Fulvio Gandolfi coordinator of PhD course in Veterinary and Animal Sciences.

I also thank Dott. Giancarlo Selmini (Ferraroni s.p.a.) for the experimental feed, Dott. Serena Reggi (Plantechno s.r.l) for the developing of specific ELISA, Dott. Loris Alborali for the challenge strain purchasing.

Thanks to Dott. Enrico Sala, Prof. Alessandra Moscatelli and Dott. ELISAbetta Onelli of the Department of Bioscience, University of Milan for the collaboration in-depth studies on the tobacco germination (Studio degli effetti dell'espressione di geni batterici sulla germinazione di semi di *Nicotiana Tabacum* by "FONDO PIANO DI SVILUPPO LINEA B).

I thankfully acknowledge Antonio Crotti, Andrea Pastori, Dott. Diego de Nisi, Dott. Raffaella Rebucci, for the support during the *in-vivo* experimentation.

A special thanks to MD Ed Janoff for hosting and welcoming me at the University of Denver, Colorado Division of Infectious Disease. Furthermore, I would like to thank Dott. Jeremy Rakhola and PhD Harsh Pratap for their backing in the lab and FACS analyses.

Lastly, I thank Dott. Raphaelle Prinsen for her help and above all for her friendship during the PhD course.