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Bioactives in milk-derived products: bacterial production of immunomodulatory casein hydrolysates and tools for identification of an immunogenic bacterial protein

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ABSTRACT

During hydrolysis of bovine milk caseins, cell envelope-associated proteinases (CEPs) of lactic acid bacteria (LAB) may be able to produce hydrolysates that possess *in vitro* immunomodulatory activity. We studied the proteolytic activity against bovine milk caseins exerted by specific strains of LAB selected on the basis of a preliminary genomic screening for the presence of CEPs. The tested strains demonstrated diverse hydrolytic ability against different casein fractions, α_{S1}- and β-casein in particular. The 3 kDa-ultrafiltered casein hydrolysates (CHs), produced after digestion with proteinases of *Lactobacillus acidophilus* ATCC 4356 and *Lactococcus lactis* subsp. *lactis* GR5 demonstrated immunomodulatory activity *in vitro* by significantly decreasing the basal NF-κB activity in recombinant Caco-2 cell layers. Both the strains digested β-casein mostly. However, in the case of *Lactobacillus helveticus* MIMLh5, the investigation proved that the immunomodulatory activity of obtained CH was comparable to the one exerted by bacteria themselves (in the absence of caseinate) and therefore, was not due to the presence of casein-derived peptides. Indeed, the surface layer (S-layer) protein of *L. helveticus* MIMLh5 was identified as a molecule responsible for the immunomodulation. Overall, these results emphasize the importance of this bacterial cell-derived protein while evaluating the immunological activity of CHs, and it underlines the necessity to remove such molecule in order to properly assess the bioactivity of CHs deriving from the proteolytic activity of LAB.

The second part of the research was focussed on the selection of surface layer (S-layer)-specific single-chain variable fragment antibodies (scFvs), a new powerful tool for the study of the S-layer of *L. helveticus* MIMLh5, the immunologically active protein, and for its identification in milk-derived products containing *L. helveticus* as a starter or non starter LAB. In this study, a mix of two human synthetic phage displayed libraries (protein-directed and hapten-directed) was used to select scFvs against *L. helveticus* MIMLh5 S-layer protein. After three rounds of panning, four monoclonal scFv binders capable of binding to the *L. helveticus* MIMLh5 S-layer protein and one capable of binding not only to the mentioned protein, but also to the S-layer protein of *L. helveticus* ATCC 15009, which is different only in five amino acids, were obtained. All five identified novel anti-S-layer scFvs were expressed in *Escherichia coli* XL1-Blue, and their basic characterisation was performed. Anti-S-layer-scFv PolyH4 was used to develop an assay, based on Western blot analysis, for detection of the S-layer protein in Grana Padano samples. These results showed promising applications of the method for the detection of the S-layer protein in food matrices.

RIASSUNTO

Composti bioattivi in prodotti derivati dal latte: idrolizzati batterici di caseina immunomodulanti e strumenti per l'identificazione di una proteina batterica con proprietà immunogeniche

Durante l'idrolisi delle caseine del latte bovino, le proteinasi della parete cellulare (CEP) di alcuni batteri lattici (LAB) possono produrre idrolizzati in grado di esplicare *in vitro* attività immunomodulante. In questo lavoro, è stata studiata l'attività proteolitica a carico delle caseine del latte bovino esercitata da specifici ceppi di LAB selezionati sulla base di un preliminare screening genomico per la presenza di CEP. I ceppi testati hanno evidenziato una diversa capacità idrolitica nei confronti delle diverse frazioni caseiniche, α_{S1}- e β- in particolare. Gli ultrafiltrati a 3 kDa degli idrolizzati caseinici (CH) prodotti dopo digestione con proteinasi di *Lactobacillus acidophilus* ATCC 4356 e *Lactococcus lactis* subsp. *lactis* GR5 hanno evidenziato attività immunomodulante *in vitro* risultando capaci di ridurre l'attività basale di NF-κB nelle cellule ricombinanti Caco-2. Entrambi i ceppi hanno evidenziato una prevalente azione idrolitica a carico della β-caseina. Tuttavia, nel caso di *Lactobacillus helveticus* MIMLh5, lo studio ha dimostrato che l'attività immunomodulante del relativo CH era paragonabile a quella esercitata dai batteri stessi (in assenza di caseina) e, quindi, non legata alla presenza di peptidi originanti dalla proteolisi della caseina. In particolare, la proteina di superficie (l'S-layer) di *L. helveticus* MIMLh5 è stata identificata come la molecola responsabile dell'attività immunomodulante del CH. Questi risultati sottolineano l'importanza di questa proteina di origine batterica durante la valutazione della attività immunologica di CH ed evidenziano la necessità di eliminare tale molecola per valutare correttamente la bioattività di CH derivati dall'azione proteolitica di LAB.

La seconda parte della ricerca è stata focalizzata sulla selezione di anticorpi fagici (*single-chain variable fragment*, scFv) specifici per l'S-layer di *L. helveticus* MIMLh5: per il suo studio e per la sua identificazione nei prodotti lattiero-caseari contenenti *L. helveticus* come colture starter o non-starter LAB. Nel dettaglio, in questo studio due librerie sintetiche umane di fagi (proteine-diretti e aptene-diretti) sono state utilizzate per selezionare scFvs specifici per la proteina S-layer di *L. helveticus* MIMLh5. Dopo tre turni di panning, sono stati identificati quattro anticorpi monoclonali scFv in grado di riconoscere la proteina S-layer di *L. helveticus* MIMLh5, ed uno in grado di riconoscere anche l'S-layer del ceppo ATCC 15009, proteina che si differenzia da quella del ceppo MIMLh5 per soli cinque aminoacidi. I cinque scFvs anti-S-layer sono stati espressi in *Escherichia coli* XL1-Blue ed è stata effettuata la loro caratterizzazione di base. Uno di essi (PolyH4) è stato utilizzato per sviluppare un test (sulla base di Western blot) per rilevare questa proteina in campioni di Grana Padano. I risultati ottenuti evidenziano promettenti applicazioni del metodo per il rilevamento della proteina S-layer in matrici alimentari.

PREFACE

There has been increased interest in the study of the relationship between nutrition and immunity due to the hypothesis that consumption of certain foods may reduce susceptibility for the establishment and/or progression of immunological diseases (Sandré et al., 2001). In the last decade, the interest in food-derived bioactive peptides has greatly increased (Korhonen & Pihlanto, 2006; Hartmann & Meisel, 2007; Phelan et al., 2009a). It is well documented that microbiological proteolysis of food proteins (milk proteins being the most widely studied) can yield physiologically important bioactive peptides (Hayes et al., 2007a; Korhonen, 2009). Bovine milk contains a number of potent immunoregulatory peptides that affect the immune system via cellular functions and there is great interest in the immunomodulatory properties of casein hydrolysates (CHs) (Migliore-Samour et al., 1989; Sandré et al., 2001; Jing & Kitts, 2004; Bennett et al., 2005; Phelan et al., 2009b).

Caseins are the most abundant milk proteins, which constitute the 78 % of total bovine milk protein content (Gagnaire et al., 2009). Enzymatic proteolysis of caseins yields many different bioactive peptides. A number of these peptides have been identified in milk CHs and fermented dairy products, as reviewed in a series of recent papers (Ferranti et al., 2004; Rowan et al., 2005; Hayes et al., 2007a, 2007b; Jensen et al., 2009; Korhonen, 2009; Phelan et al., 2009a, 2009b). They have been shown to exert both functional and physiological roles *in vitro* and *in vivo*, and because of this they are of particular interest for food science and nutrition applications. Biological activities associated with such peptides include: immunomodulatory, cytomodulatory, hypotensive, antimicrobial, mineral binding, antioxidant, antithrombotic along with opioid agonist and antagonist activities (Gill et al., 2000; Korhonen & Pihlanto, 2006; Korhonen, 2009; Phelan et al., 2009a).

These milk casein-derived biologically active peptides are inactive within the sequence of a parent protein, but can be released during gastrointestinal digestion, food processing and fermentation. The latter comprehends the hydrolysis by proteolytic starter or non-starter microorganisms such as lactic acid bacteria (LAB) (Phelan et al., 2009a; Tellez et al., 2010, 2011).

Therefore, the first part of this PhD research was directed towards the investigation of the immunomodulatory activity of bovine casein hydrolysates produced after digestion with proteinases of LAB.

Many LAB have a complex proteolytic system capable of degrading caseins (Jensen et al., 2009; Sadat-Mekmene et al., 2011) and thus not only having a strong impact on texture and flavour of cheese in its production chain (Oberg et al., 1991; Smit et al., 2005), but also producing bioactive peptides including those with immunomodulatory properties (Matar et al., 2001; LeBlanc et al., 2002; LeBlanc et al., 2004; Tellez et al., 2010, 2011). On the other hand, LAB such as *Lactobacillus helveticus*, a thermophilic homofermentative LAB traditionally used in cheese manufacture (Fortina et al., 1998; Gatti et al., 2004; Jensen et al., 2009), can exert their bioactive abilities by themselves, influencing the host's immune system (Vinderola et al., 2005; Galdeano et al., 2007; Timmerman et al., 2007; Lebeer et al., 2008; Guglielmetti et al., 2010; O'Flaherty & Klaenhammer, 2010).

It was demonstrated that surface-located molecules, such as heat-shock protein rGroEL (Bergonzelli et al., 2006) and surface layer (S-layer) protein (the most abundant bacterial cell wall protein, Beganović et al., 2011; Taverniti & Guglielmetti, 2011; Taverniti et al., manuscript in preparation), contribute to this activity. Thus, in the light of immunomodulation,

the presence of starter and non-starter bacterial cultures should be considered in fermented milk products while evaluating their immunomodulatory properties. Furthermore, the necessity to have tools for the study of the immunologically active S-layer protein and for its identification in milk-derived products containing *L. helveticus* as a starter or non starter LAB appeared.

Therefore, the second part of this PhD research was focussed on the development of protein-specific binders by increasingly important phage display technology (Willats, 2002; Bradbury & Marks, 2004; Kehoe & Kay, 2005; Bradbury et al., 2011), a new powerful tool for the study of *L. helveticus* S-layer protein and for its identification in milk-derived products containing *L. helveticus*.

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1 IMMUNOMODULATORY ACTIVITY OF BOVINE CASEIN HYDROLYSATES PRODUCED AFTER DIGESTION WITH CELL ENVELOPE-ASSOCIATED PROTEINASES OF LACTIC ACID BACTERIA

1.1 STATE OF THE ART

1.1.1 Biologically active peptides: the definition

Bioactive peptides are described as 'food-derived components (native or generated) that in addition to their nutritional value, exert a physiological effect in the body' (Vermeirssen et al., 2004). The definition of bioactive peptides include peptides generated from food, which can affect one or more biological processes causing a measurable effect on the functions and conditions of our body and, ultimately, on health. This definition implies the exclusion of peptides with toxic and allergenic effects (Schrezenmeir et at., 2000).

Today, milk proteins are considered the most important source of bioactive peptides and an increasing number of bioactive peptides have been identified in milk protein hydrolysates and fermented dairy products. Bioactive peptides derived from milk proteins offer a promising approach for the promotion of health by means of a tailored diet and provide interesting opportunities to the dairy industry for expansion of its field of operation (Nagpal et al., 2011).

1.1.2 Production mechanisms of biopeptides: focus on the proteolytic system action of LAB

Milk proteins may contain several biologically active peptides with sizes varying from 2 to 40 amino acids (Korhonen, 2009; Phelan et al., 2009a). They are inactive within the sequence of a parent protein, but can be released during an enzymatic pre-digestion: (i) gastrointestinal digestion: hydrolysis with proteolytic enzymes secreted into the gastrointestinal tract such as pepsin and chymotrypsin, (ii) food processing: industrial food manufacturing processes, including biotransformation with isolated enzymes, and (iii) fermentation: microbial enzymes expressed by microorganisms of the intestinal and food microbiota (Möller et al., 2008; Korhonen, 2009).

A large variety of LAB are widely used as starters to produce fermented dairy products. They are quite fastidious microorganisms and require an exogenous source of amino acids or peptides for optimal growth. As milk is poor in these low-molecular-weight compounds, its growth largely depends on their proteolytic system to achieve hydrolysis of caseins into peptides and amino acids (Thomas & Pritchard, 1987). The enzymes involved in milk protein degradation are: (i) cell envelope proteinases (CEPs), and (ii) intracellular peptidases (Thomas & Pritchard, 1987). CEPs are the key enzymes of this process since they are the only enzymes responsible for the first step of milk casein breakdown into oligopeptides (Siezen, 1999). The latter are then transported into the bacteria by a transport system for amino acids, and di-, tri- and oligopeptides and further degraded by a complex set of intracellular peptidases (Christensen et al., 1999).

Moreover, CEPs of intestinal microbiota achieve hydrolysis of caseins into peptides with biological activities (Hayes et al., 2007a, 2007b). Also in the case of immunomodulatory peptides, milk fermentation contributes to the generation of fermented milk with potential immunological activity. Despite detailed knowledge of the proteolytic systems of LAB as outlined above, information on the production of bioactive peptides through milk fermentation and the specific proteinases responsible for bioactive peptide release during dairy fermentation is lacking, with only a few reports available (Hayes et al., 2007a)

The cell wall proteinase of *Lactococcus lactis* was shown to *in vitro* liberate oligopeptides from β- and α-caseins, which contain amino acid sequences present in casomorphins, casokinines, and immunopeptides (Meisel & Bockelmann, 1999). The further degradation of these peptides by endopeptidases and exopeptidases of LAB could lead to the liberation of bioactive peptides in fermented milk products. Algaron et al. (2004) fermented milk with mutant *L. lactis* strains

lacking either aminopeptidase N, PepX or tripeptidase to test their ability to form peptides with anti-hypertensive or immunomodulatory activities, and to relate the resultant bioactivity to a specific mutation. Authors reported that, in some cases, the modified proteolytic system of *L. lactis* gave rise to a significant difference in the mixture of peptides produced during milk fermentation, indicating that the specific peptidase activity of LAB affects the bioactive nature of the peptides produced.

In *Streptococcus thermophilus*, the presence of a CEP, PrtS, is less common than in *L. lactis*. In *S. thermophilus*, high CEP activities are associated with high milk-acidifying capacities (Shahbal et al., 1991). Strain CNRZ 385 of this species was first characterised by its rapid growth and high acidification rate in milk (Shahbal et al., 1991). This rapid growth was correlated with the presence of high proteinase activity associated with the cell wall of *S. thermophilus* strains. Thus, the main role of PrtS was concluded to concern the amino acid supply to the cell via casein hydrolysis. This proteinase was later purified and characterised biochemically as well as genetically (Fernandez-Espla et al., 2000). In addition, *S. thermophilus* cell wall proteinases were proposed be involved in the development of dairy product health properties via bioactive peptide production (Fernandez-Espla et al., 2000). However, no direct experimental data exist up to date. MacDonald et al. (1994) and Ganjam et al. (1997) demonstrated that bioactive peptides present in yoghurt fermented with *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, decreased cell proliferation of IEC-6 or Caco-2 human intestinal epithelial cell lines, which may explain, at least partially, why consumption of yoghurt has been associated with a reduced incidence of colon cancer. De Simone and collaborators (De Simone et al., 1986) tested interferon γ production of human peripheral blood lymphocytes in response to filtered yoghurt devoid of microorganisms, which lets the assumption that the immunomodulatory activity is independent from the presence of living microorganisms.

In general, *L. helveticus* is known to have high proteolytic activity, causing the release of oligopeptides from digestion of milk proteins (Lozo et al., 2011; Sadat-Mekmene et al., 2011). Laffineur and colleagues (1996) showed that β-casein, fermented with *L. helveticus*, modulates lymphocyte proliferation *in vitro*. The same bacterial species, selected for the ability to produce fermented milk with high ACE-inhibitory activity, produces a fermented milk with immunomodulatory properties. Rachid et al. (2006) demonstrated that the administration of milk fermented with *L. helveticus* decreases the growth rate of tumours in a murine model for mammary carcinoma. In addition, LeBlanc et al. (2002) used the strain *L. helveticus* R389 to ferment milk. The fermented milk was administered to mice with fibrosarcoma, resulting in a decrease of tumour size. Moreover, Matar and colleagues (2001) tested the *L. helveticus* R389 non-proteolytic variant for the ability to produce peptides with immunomodulatory activities. They reported that protease-deficient variant of *L. helveticus* R389 failed to induce a general activation of the immune system, demonstrating the importance of the CEP activity of the strain in the production of peptides with investigated biological activity.

Milk fermented by *L. helveticus* not only demonstrated anti-tumoural properties but also induced the total antibody production against *E. coli* O157:H7 in mice infected with this pathogen (LeBlanc et al., 2002; LeBlanc et al., 2004).

Besides the *L. helveticus*, other LAB were demonstrated to produce milk protein hydrolysates with immunomodulatory properties. In fact, milk fermented with *Lactobacillus paracasei* was shown to produce peptides from β-lactoglobulin that stimulate IL-10 production and depress the lymphocyte proliferation (Prioult et al., 2004). Sütas and co-workers (1996a, 1996c) demonstrated that caseins hydrolysed by *Lactobacillus casei* GG and digestive enzymes

generated compounds with suppressive effects on human T lymphocyte activation, modulating IL-2 expression.

The immunomodulatory activity is independent from the presence of living *L. helveticus*, as demonstrated by LeBlanc and collaborators (2004) who examined the antibody production after *E. coli* O157:H7 infection following the administration of a cell-free supernatant from *L. helveticus* fermented milk. They found that the increased antibody production is not related to viable microorganism. More recently, Vinderola et al. (2007) showed that the supernatant of fermented milk cultured with *L. helveticus* increased the immune response independently from the presence of lactobacilli.

Undoubtedly, identification of the links between the proteolytic machinery of cultures used in dairy fermentation and resulting bioactivity profiles of milk will benefit the development of 'designer' milk fermentations with specific, tailored and proven health promoting compounds targeted at niche sections of the food and possibly pharmaceutical industries (Hayes et al., 2007a).

1.1.3 Accessibility of biopeptides by human host

Only some of the generated biopeptides can cross the intestinal barrier (Hara et al., 1984) and reach the target while maintaining their functionality. These peptides have to be small (maximum 3 amino acids) in order to cross the enterocyte barrier and must be able to resist complete digestion by peptidases found in the cytoplasm of enterocytes. The resistance to these enzymes is conferred by the presence in the amino acid sequence residues of proline (Pro), a sterically bulky amino acid that makes difficult the access of the peptide bond for hydrolytic enzymes (Korhonen & Pihlanto, 2006). An example is the hypotensive tripeptide Val-Pro-Pro, whose target is the angiotensin converting enzyme (ACE). It was demonstrated that this peptide is able to cross the Caco-2 cell layer in significant quantities, mainly through a paracellular diffusion mechanism (Satake et al., 2002). A different mode of transport has been demonstrated for tripeptide Lys-Pro-Val, derived from α-melanocyte-stimulating hormone (α-MSH), which is able to inhibit the transcriptional activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in Caco-2 cells in the presence of a transporter of small peptides, called hPepT1, expressed on the apical membrane of intestinal cells (Dalmasso et al., 2008), suggesting that the mechanism of action of bioactive peptides may involve intracellular target.

Peptides with dimensions that do not allow absorption through the intestinal barrier, may act only inside the lumen, where they can modulate the activity of receptors located on the apical membrane of enterocytes or be "sampled" by dendritic cells, which are in close contact with the lamina propria of the intestine (Rescigno et al., 2001).

1.1.4 Milk protein hydrolysates as a source of biologically active peptides

The rich source of proteins and bioactive peptides is represented by milk and its derivatives, by virtue of its role in health protection of a newborn mammal and in the development of his immunity (Tsopmo et al., 2009; Szwajkowska et al., 2011). The milk is constituted by a high protein content, represented by 78 % of the different casein isoforms (α_{S1}, α_{S2}, β and κ; Gagnaire et al., 2009); the rest part is constituted of whey proteins (mainly β-lactoglobulin and α-lactalbumin), growth factors, lactoferrin, immunoglobulins and other proteins (Table 1.1.1).

Table 1.1.1. *Concentration and biological activity of major bovine milk proteins (adapted from Mills et al., 2011).*

Protein	Concentration ($g\ L^{-1}$)	Function
Total caseins	26.0	Ion carrier (Ca, PO_4, Fe, Zn, Cu), precursor of bioactive peptides
α-caseins	13.0	Precursor of bioactive peptides
β-caseins	9.3	Precursor of bioactive peptides
κ-casein	3.3	Precursor of bioactive peptides
Total whey protein	6.3	Anticarcinogenic, weight management
β-Lactoglobulin	3.2	Retinol carrier, binding fatty acids, possible antioxidant
α-Lactalbumin	1.2	Lactose synthesis in mammary gland, Ca carrier, immunomodulation
Immunoglobulins (A, M, and G)	0.7	Immune protection
Serum albumin	0.4	
Lactoferrin	0.1	Antimicrobial, antioxidative, immunomodulation, iron absorption
Lactoperoxidase	0.003	Antimicrobial
Lysozyme	0.0004	Antimicrobial, synergistic effect with immunoglobulins and lactoferrin
Miscellaneous	0.8	
Proteose–peptone	1.2	Function unknown, but precursor of bioactive protein and peptide *in vitro*
Glycomacropeptide	1.2	Antiviral, bifidogenic

A variety of milk protein-derived biologically active peptides have been shown to exert both functional and physiological roles *in vitro* and *in vivo*, and because of this they are of particular interest for food science and nutrition applications. Biological activities associated with such peptides include: antibacterial, hypotensive, immunomodulatory, mineral binding, antithrombotic along with opioid agonist and antagonist activities (Korhonen & Philanto, 2006; Phelan et al., 2009a, 2009b).

The milk protein-derived bioactive peptides can be used in production of functional foods, foods with scientifically proved beneficial properties. In recent years, the market demand for these products has increased, especially in Europe, Japan and Australia (Hilliam, 2003). The development and marketing of functional foods requires not only understanding of the detailed mechanisms of action and possible side effects, but also aspects related to the technological and industrial-scale production.

1.1.5 Casein-derived biopeptides: focus on peptides displaying immunomodulatory activity

Casein is the main proteinaceous component of milk. Formerly the main physiological role of casein in the milk system was widely accepted to be a source of amino acids required by growth of the neonate. However, the dominant physiological feature of the casein micelle system was later proven to be the prevention of pathological calcification of the mammary gland (Holt, 1997). While no specific physiological property has been proposed for the whole casein system (or its individual fractions), various peptides hidden (or inactive) in the amino-acid sequence

have been the subject of increasingly intense studies (Phelan et al., 2009a; Silva & Malcata, 2005).

A number of potentially immunoregulatory peptides were identified encrypted in caseins (Table 1.1.2).

Table 1.1.2. *Immunomodulatory peptides derived from milk caseins (adapted from Silva & Malcata, 2005, Hayes et al., 2007b, and Nagpal et al., 2011).*

Precursor protein	Fragment	Peptide sequence	Name	Reference
α_{S1}-casein (bovine)	1–23	RPKHPIKHQGLPQE VLNENLLRF	isracidin	Lahov & Regelson, 1996 Minkiewicz et al., 2000
	23–34	FFVAPFPEVPGK		Maruyama et al., 1987 Jollés et al., 1992
	90–96	RYLGYLE		Migliore-Samour et al., 1989 Elitsur & Luk, 1991
	90–95	RYLGYL		Migliore-Samour et al., 1989 Elitsur & Luk, 1991
	194–199	TTMPLW	α-caso kinin-6	Parker et al., 1984 Migliore-Samour & Jollés, 1988 Hernandez-Ledesma et al., 2004
α_{S2}-casein (bovine)	1–32	KNTMEHVSSSEESII SQETYKQEKNMAI NPSK		Coste et al., 1992 Hata et al., 1999 Dionysius et al., 2000
β-casein (bovine)	1–28	RELEELNVPGEIVE SLSSSEESITRINK		Coste et al., 1992 Hata et al., 1999 Dionysius et al., 2000
	60–66	YPFPGPI	β-caso morphin-7	Maruyama et al., 1987 Elitsur & Kuk, 1991 Kayser & Meisel, 1996
	63–68	PGPIPN		Parker et al., 1984 Migliore-Samour & Jollés, 1988 Coste et al., 1992 Fiat et al., 1993 Dionysius et al., 2000
	191–193	LLY		Parker et al., 1984 Migliore-Samour & Jollés, 1988 Coste et al., 1992 Fiat et al., 1993 Dionysius et al., 2000
	191–209	LLYQEPVLGPVRGP FPIIV		Coste et al., 1992
	193–202	YQQPVLGPVR	β-caso kinin-10	Maruyama et al., 1987 Kayser & Meisel, 1996

Precursor protein	Fragment	Peptide sequence	Name	Reference
	193–209	YQEPVLGPVRGPFP IIV		Minkiewicz et al., 2000 Sandré at al., 2001
	145–160	HQPHQPLPPTVMFP PQ		Tellez et al., 2010 Tellez et al., 2011
	143–154	WMHQPHQPLPPT		
	145–154	HQPHQPLPPT		
	192–202	LYQEPVLGPVR		
β-casein (human)	54–59	VEPIPY		Parker et al., 1984 Migliore-Samour et al., 1989 Coste et al., 1992
κ-casein (bovine)	38–39	YG		Kayser & Meisel, 1996
	106–169	MAIPPKKNQDKTEI PTINTIASGEPTSTP TTEAVESTVATLED SPEVIESPPEINTVQ VTSTAV	Glycomac-ropeptide	Otani & Hata, 1995 Otani et al., 1995

These milk casein-derived immunomodulatory peptides have been shown to modulate various aspects of immune function (Gill et al., 2000).

Immunomodulatory casein-derived (and in general milk-derived) peptides may contribute to the overall immune response and may improve the immune system function. It was suggested that casein-derived peptides are involved in the stimulation of the newborn's immune system and probably even have a direct effect on the resistance to bacterial and viral infection of adult humans (Migliore-Samour et al., 1989).

The evaluation of immunomodulatory activity of casein-derived immunoactive peptides was made by measuring the proliferation of lymphocytes, the secretion of immunoglobulin (Ig), IgA in particular, the phagocytic activity of macrophages and the effect on cytokine expression.

Casein-derived peptides showed the ability to modulate the proliferation of human lymphocytes (Möller et al., 2008). It was manifested that peptides derived from whole casein or α_{S1}-casein limit the proliferation of human lymphocytes, whereas those derived from β- and κ-casein have, generally, the opposite effect (Korhonen & Philanto, 2003a, 2003b).

It was also demonstrated that peptides derived from α_{S1}-, β- and κ-casein have been shown to both stimulate and suppress lymphocyte proliferation (Migliore-Samour & Jollés, 1988; Elitsur & Luk, 1991; Otani & Hata, 1995; Sütas et al., 1996a, 1996b, 1996c). Certain peptides derived from α_{S1}-casein and β-casein have been reported to suppress mitogen-induced proliferation of human lymphocytes and peripheral blood mononuclear cells while stimulating lymphocyte proliferation in the absence of mitogens (Kayser & Meisel, 1996). Other fragments (fragment 18–20 of κ-casein, fragment 90–96 of α_{S1}-casein) can either stimulate or inhibit lymphocyte proliferation depending upon the concentration used (Migliore-Samour et al., 1989; Kayser & Meisel, 1996). Maruyama et al. (1987) and later Kayser & Meisel (1996) reported that, in cell culture model systems, β-casomorphin-7 and β-casokinin-10 were immunoenhancing at high concentrations and immunosuppressing at low concentrations.

It was demonstrated that glycomacropeptide, a κ-casein derivative, inhibits the proliferation of splenocytes of mice (Otani & Hata, 1995) and enhances IgA production (Yun et al., 1996).

Peptides derived from the fermentation of milk with *L. helveticus* showed the ability to stimulate proliferation of lymphocytes and phagocytic activity of pulmonary macrophages (Laffineur et al., 1996). The observations *in vitro* were confirmed by *in vivo* studies in mice: the administration of *L. helveticus* hydrolysates, followed by infection with *E. coli* caused an increase of IgA-secreting B cells. Instead, this effect was not observed by administering the hydrolysis product of a genetically modified, protease-negative, strain of *L. helveticus*, indicating an effect closely related to the proteolytic activity of the microorganism (LeBlanc et al., 2002).

Some casein-derived peptides (residues 54–59 of human β-casein and residues 194–199 of bovine α_{S1}-casein) can stimulate phagocytosis of sheep red blood cells by murine peritoneal macrophages (Parker et al., 1984; Jollés et al., 1992), exert a protective effect against *Klebsiella pneumoniae* (Migliore-Samour et al., 1989) or modulate immunoglobulin production in mouse spleen cell cultures (fragment 1–28 of bovine β-casein, (Otani & Hata, 1995; Otani et al., 2001).

CHs have also been shown to have an effect on cytokine expression (Phelan et al., 2009a). Phelan et al. (2009b) demonstrated that CHs generated a Th1 response by enhancing ConA-induced interleukin-2 (IL-2) production while having no effect on IL-10. Kawahara & Otani (2004) analysed the effect of the commercially available caseinophosphopeptide preparation CPP-III, consisting mainly of the fragments α_{S2}-CN (1–32) and β-CN (1–28) from bovine caseins, on the mRNA expression of cytokines in Caco-2 cells. CPP-III enhanced mRNA expression of IL-6 and tumour necrosis factor-α (TNF-α), while IL-1β was not affected. mRNA expression of IL-6 was stronger in the presence of both CPPs-III and peptidoglycan from *Lactobacillus acidophilus* and lipopolysaccharide (LPS) from *Salmonella typhimurium*. Kitts & Nakamura (2006) showed that only one of the three analysed CPPs (CPPs-III) stimulated IL-6-induced immunostimulation in intestinal epithelial-like cells Int-407. In vitro results suggest that CHs mainly affect the production of Th1 cytokines and, hence, have a role in cellular immunity (Phelan et al., 2009a).

However, the mechanisms by which these milk-derived peptides exert their immunomodulatory effects or influence cell proliferation are not currently fully understood (Phelan et al., 2009a).

Several immunomodulatory peptides are multifunctional peptides. Some of them may modulate cell proliferation via ACE-inhibitory mechanism. ACE-inhibitory peptides are known for their hypotensive properties, because they inhibit the conversion of angiotensin I to angiotensin II, but have also been found to prevent cleavage of bradykinin, that is mediated by ACE (Jollés et al., 1992). Bradykinin acts as a mediator of the acute inflammatory process and is thus able to enhance lymphocyte migration, stimulate macrophages, and induce the secretion of cytokines from lymphocytes in culture. It should be noted that a common structural feature of several ACE-inhibitory peptides and some immunomodulatory peptides is the presence of arginine as the C-terminal residue (Nagpal et al., 2011).

The other example of multifunctional peptides is the opioid peptide β-casomorphin, derived from human β-casein. It inhibits *in vitro* the proliferation of human lamina propria lymphocytes via opiate receptor (Elitsur & Kuk, 1991). On the other hand, opioid peptides stimulate the maturation and proliferation of T cells and natural killer cells (Rutherfurd-Markwick & Moughan, 2005). Indeed, the immune system and opioid peptides are related: it has already

been demonstrated that opioid receptors are expressed on T lymphocytes and human phagocytic leukocytes (Meisel, 1998; Carr et al., 1989).

It has been suggested that arginine in the N- or C-terminal region of a peptide is an important structural entity recognised by specific membrane bound receptors. A common structural feature among some immunomodulatory peptides is the presence of arginine in the C-terminal (Meisel, 1998; Nagpal et al., 2011).

Caseinophosphopeptides, other class of casein-derived peptides with immunomodulatory activity, CPP-III in particular, enhance the proliferative response induced by LPS, phytohaemagglutinin and concanavalin A (ConA) stimulation, and immunoglobulin production in mouse splenocyte cultures (Hata et al., 1998; Hata et al., 1999). This immunostimulating activity was attributed to the o-phospho-L-serine residue, suggesting that such a bioactivity is relatively stable to proteinase action in the intestinal tract (Otani et al., 2000). The research paper of Otani et al. (Otani et al., 2003) focusing on the effects of CPP-III on serum and intestinal Ig G and Ig A secretion in mice, proved that oral use of CPP-III is beneficial toward enhancement of the mucosal immunity.

1.2 AIMS OF THE STUDY

Bovine milk contains a number of potent immunoregulatory peptides that affect the immune system via cellular functions and there is great interest in the immunomodulatory properties of casein hydrolysates (CHs) (Eriksen et al., 2008; Phelan et al., 2009a, 2009b). Hydrolysis of bovine milk caseins by CEPs of LAB can yield a number of immunologically active biopeptides, as it was mentioned above in the literature review. However, until recently little was known about interactions of these peptides with intestinal mucosae at the immunological level (Korhonen & Pihlanto, 2006; Phelan et al., 2009b).

Therefore, the aim of the present study was to determine *in vitro* the immunomodulatory activity at the enterocyte level of bovine CHs produced by digestion with CEPs of LAB.

Most specifically the tasks were:

1. Examine proteolysis of bovine caseinate by CEP-positive LAB.
2. Develop the system based on the response of intestinal epithelial-like Caco-2 cells and determine immunomodulatory activity *in vitro* of CHs, as a source of potential biologically active peptides.

1.3 MATERIALS AND METHODS

1.3.1 Bacterial strains and culture conditions

Table 1.3.1 lists the bacterial strains used in this study. *S. thermophilus* strains were grown at 37 °C in M17 medium (Sigma–Aldrich, Milan, Italy) supplemented with 20 g of lactose liter^{-1}. *L. lactis* subsp. *lactis* was cultivated in M17 medium supplemented with 0.5 % of lactose and 0.5 % of glucose at 30 °C. *L. helveticus* and *L. acidophilus* were grown in MRS broth (Sigma–Aldrich) supplemented with 1 % of Tween 80 (Sigma–Aldrich) at 37 °C.

Table 1.3.1. *Bacterial strains included in the study.*

Species	Strain	Source
Streptococcus thermophilus	3207[a]	Yogurt obtained in Georgia-Kutaisi
	CNRZ 385[b]	Japanese yogurt (Yakult Company)
	DSM 20617[T]	Pasteurized milk
Lactobacillus helveticus	MIMLh5[a]	Grana Padano cheese natural whey starter
	Lh164[a]	Natural whey starter
	ATCC 15009[T]	Emmental (Swiss) cheese
Lactobacillus acidophilus	La-5[c]	Commercial probiotic strain
	LA03[d]	Commercial probiotic strain
	ATCC 4356[T]	Human
Lactococcus lactis subsp. *lactis*	GR1[a]	Sour cream obtained in Lithuania
	GR5[a]	Sour cream obtained in Lithuania

[a] DiSTAM Industrial Microbiology Section Culture Collection, University of Milan (Milan, Italy).
[b] LAB collection of the Institut National de la Recherche Agronomique (Jouy-en-Josas, France).
[c] Chr. Hansen A/S Culture Collection (Horsholm, Denmark).
[d] Sacco Culture Collection (Cadorago, Italy).
[T] Type strains from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and American Type Culture Collection (Manassas, VA, USA).

1.3.2 Determination of CEP (*prt*) gene fragments

Determination of CEP gene (*prtS* for *S. thermophilus*, *prtH* and *prtH2* for *L. helveticus*, *prtR* for *L. acidophilus* and *prtP* for *L. lactis* subsp. *lactis*) fragments was performed by PCR using specific primers designed for the internal part of the protease genes (Table 1.3.2) on the DNA extracted from the isolates according the standard procedures (Sambrook et al., 2001).

Table 1.3.2. *Primers used in this study.*

Primer	Sequence (5' to 3')	Size of PCR product (bp)	Reference
PrtSf	TGTTCCTGACGCAATCGTTCA	680	This study
PrtSr	CGTGCATACCGTGTGAACGT		
PrtH2-for-3	GTTGGTGCCGCAACTAAATC	430	Genay et al., 2009
PrtH2-rev-2	TAGCATTTTGGTCAAAGACA		
PrtH-for-1	GGTACTTCAATGGCTTCTCC	470	Genay et al., 2009
PrtH-rev-1	GATGCGCCATCAATCTTCTT		
PrtRf	GTTGGTGCAGCAACTAAAGC	427	This study
PrtRr	TAGCTTTCTTGTCAAAGACA		

Primer	Sequence (5' to 3')	Size of PCR product (bp)	Reference
P15C	AACCAAATCTGATGTTG	560	Nikolic et al., 2009
P06C	TTTCAGCGGAAGCAACT		

The PCR conditions were as follows: predenaturation at 95 °C for 4 min, 30 cycles at 95 °C for 30 s, 61 °C (for *prtS*), 58 °C (for *prtH*2, *prtH* and *prtR*), 55 °C (for *prtP*) for 30 s, 72 °C for 1 min, and a single final extension at 72 °C for 7 min. All amplification reactions were performed in a MyCycler thermal cycler (BioRad Italia, Milan, Italy).

1.3.3 Preparation of CHs

Sodium caseinate was prepared from unprocessed bovine bulk milk. This milk was firstly skimmed and subjected to sodium caseinate preparation according to Hammarsten (1882–1883). Bacterial strains were subjected to evaluation of sodium caseinate hydrolysis by CEPs as described by Arioli et al. (2007) and Hebert et al. (2008) with slight modifications. Briefly, overnight culture was harvested by centrifugation, washed twice with and resuspended in 0.85 % (w/v) NaCl solution. Cell suspensions were incubated for 30 min at bacterial growth temperature before casein degradation was carried out (in order to consume residual amino acids and peptides), washed again with 0.85 % (w/v) NaCl solution and resuspended to a final OD_{600} of approximately 10 (which corresponded to approximately 10^{10} cells mL^{-1}) in 0.1 M TRIS–HCl buffer (pH 8.0). Cell suspension was mixed with 10 mg mL^{-1} of sodium caseinate dissolved in 0.1 M TRIS–HCl (pH 8.0) at a ratio 9:1 (v/v). The resulting mixtures were incubated at bacterial growth temperature with slight agitation (140 rpm). After 5 h samples were centrifuged (8500 × g 4 °C for 10 min), filtered through a 0.2-μm-pore-size sterile cellulose acetate membrane filter (Albet-Hahnemuehle, Barcelona, Spain) and subjected to ultrafiltration and reverse phase high-performance liquid chromatography (RP–HPLC) analysis. Peptides of less than 3 kDa were separated from whole CHs using ultrafiltration through Ultracel regenerated cellulose ultrafiltration membrane NMWL 3000 (Millipore, Vimodrone, Italy).

1.3.4 Evaluation of sodium caseinate hydrolysis by RP–HPLC

Before analysis, samples were diluted 1:4 with a solution containing 8 M urea and 0.1 % trifluoroacetic acid (TFA) in MilliQ-treated water (Millipore). After resting at room temperature for at least 15 min, samples were analyzed by RP–HPLC on Waters Alliance 2695 instrument (Waters, Vimodrone, Italy). Proteins and peptides were separated at 40 °C on a PLRP-S column (2.1 mm i.d., 5 μm, 300 Å, Polymer Laboratories Ltd, Church Stretton, UK). The eluents used for the separation were: solvent A, 0.1 % TFA in MilliQ-treated water, and solvent B, 0.1 % TFA in acetonitrile. The linear elution gradient expressed as the solvent B proportion was as follows: 0 min, 30 %; 0 to 5 min, 30 %; 5 to 40 min, 30 to 50 %; 40 to 41 min, 50 to 95 %; 41 to 45 min, 95 %; 45 to 46 min, 95 to 30 % (run-to-run time, 50 min). Proteins and peptides were eluted at a flow rate of 0.2 mL min^{-1} and monitored at 210 nm.

1.3.5 Cultivation of Caco-2 cells

Caco-2 cells were routinely grown as previously explained (Guglielmetti et al., 2008). In brief, Caco-2 were cultivated in 9-cm diameter Petri plates in Eagle's minimum essential medium supplemented with 10 % (v/v) heat-inactivated (30 min at 56 °C) foetal calf serum, 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin, 0.1 mM nonessential amino acids, and 2 mM L-glutamine (EMEM) and incubated at 37 °C in a water-jacketed incubator in an atmosphere of 95 % air and 5 % carbon dioxide. The culture medium was changed twice weekly.

1.3.6 Construction of stable NF-κB reporting Caco-2 cells

Stable transfectants of Caco-2 cell line were obtained after transfection with the plasmid pNiFty2-Luc (Figure 1.3.1; InvivoGen, Labogen, Rho, Italy).

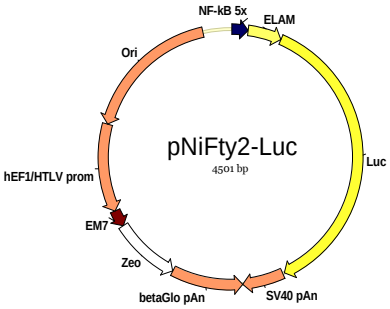

Figure 1.3.1. *Genetic map of a plasmid pNiFty2-Luc.*

This plasmid contains promoter combining five NF-κB-binding sites and the firefly luciferase reporter gene luc. The presence of NF-κB activating molecules in the cell activates this transcription factor, which binds to the promoter, resulting in the expression of the luciferase gene.

Transfection was performed and stable recombinant clones were selected as described in Guglielmetti et al. (2010a). Briefly, Caco-2 were transfected by means of the StoS transfection kit (GeneSpin, Milan, Italy), in accordance with the manufacturer's protocol. Afterward, cells were resuspended in fresh EMEM, seeded in 24-well plates, and incubated for 48 h, in order to obtain the expression of the antibiotic resistance. Finally, stable recombinant clones were selected by adding into the culture medium 50 μg ml^{-1} of zeocin (InvivoGen).

1.3.7 Evaluation of immunomodulatory activity of CHs

Recombinant Caco-2 cells were cultured in 24-well plates in presence of 50 μg mL^{-1} zeocin. Cell monolayers (approximately 3×10^5 cells $well^{-1}$) were carefully washed with 0.1 M TRIS-HCl buffer (pH 8.0), and 1.35 mL of fresh EMEM medium, containing 25 mM of HEPES (pH 7.4), was added. Subsequently 0.15 mL of CHs (which correspond to 150 μg of hydrolysed sodium caseinate) were added into each well. After incubation at 37 °C for 4 h, 24-well plates were put on ice for 15 min; recombinant Caco-2 cells were detached mechanically from the bottom of a well, samples were transferred into an eppendorf tube and subjected to sonication at maximum power for 5 s using a Bandelin SONOPLUS Ultrasonic Homogenizer (Bandelin electronic GmbH & Co., Berlin, Germany). Insoluble particles were removed by centrifugation and the supernatants were transferred into a new tube. One hundred μL of supernatants were aliquoted in duplicate into the wells of a 96-well white microtiter plate (PerkinElmer, Monza, Italy) by means of ep*Motion* Automated Pipetting System (Eppendorf, Milan, Italy). Then 12.5 μL of a 10 mM ATP solution (i.e. up to the final concentration of 1 mM) and 12.5 μL of 0.1 mM D-luciferin were added and the emitted bioluminescence was immediately measured every 120 s with a VICTOR^3 1420 Multilabel Counter (PerkinElmer). The maximum of light-production curve was considered for comparison of results. In a different set of experiments, recombinant Caco-2 cells were simultaneously stimulated with IL-1β (2 ng mL^{-1}). All CHs were analysed in duplicate in at least three independent experiments.

1.3.8 Statistical analysis

The significance of the results in the experiments for the evaluation of immunomodulatory activity of CHs was analysed by unpaired heteroscedastic Student's *t* test with two-tailed distribution. Differences of $P < 0.05$ were considered to be significant.

1.4 RESULTS AND DISCUSSION

1.4.1 Proteolytic activities of lactic acid bacterial strains

The CEPs of LAB substantiate food fermentation process and ensure bacterial growth (Christensen et al., 1999; Siezen, 1999). Moreover, they have the ability to liberate from food proteins a large variety of peptides with potential biological activities (Hebert et al., 2008; Hernandez-Ledesma et al., 2004). Therefore, eleven different LAB strains were selected for analysis: 3 belonging to the species *S. thermophilus* (3207, CNRZ 385, DSM 20617), 3 to the species *L. helveticus* (MIMLh5, Lh164, ATCC 15009), 3 to the species *L. acidophilus* (La-5, LA03, ATCC 4356), and 2 *L. lactis* subsp. *lactis* strains (GR1, GR5).

S. thermophilus CNRZ 385 possesses a proteolytic activity and its CEP (called PrtS) has been extensively characterised (Shahbal et al., 1991; Shahbal et al., 1993; Fernandez-Espla et al., 2000; Courtin et al., 2002). Also, it was demonstrated that *L. helveticus* ATCC 15009 has cell surface proteinase activity, as measured by the hydrolysis of fragment f1-23 of α_{S1}-casein (Pederson et al., 1999; Jensen et al., 2009). Moreover, caseinolytic properties of dairy *L. helveticus* and *L. lactis* strains are well documented (Jensen et al., 2009; Nikolic et al., 2009). Using specific primers designed for the internal part of the protease genes (Table 1.3.2), in PCR reactions on the DNA extracted from the isolates we obtained the expected length PCR fragments for bacterial CEP genes in six other analysed LAB strains: *prtS* in *S. thermophilus* 3207, *prtH2* in *L. helveticus* MIMLh5 and Lh164, *prtR* in *L. acidophilus* LA03 and ATCC 4356, and *prtP* in *L. lactis* subsp. *lactis* GR5, and confirmed the presence of *prtS* in *S. thermophilus* CNRZ 385 and *prtH2* in *L. helveticus* ATCC 15009. *L. acidophilus* La-5 and *L. lactis* subsp. *lactis* GR1 were protease-negative. *S. thermophilus* DSM 20617 was selected as a protease-negative control (Arioli et al., 2007).

All eight CEP-positive strains were subjected to sodium caseinate hydrolysis with the scope to obtain hydrolysates with immunomodulatory activities. Hydrolysis profiles, paying the largest attention for the hydrolysis of α_{S1} and β caseins as the major potential source of bioactive peptides with immunomodulatory activities (for a recent review see Phelan et al., 2009a), were analysed by RP–HPLC (Figure 1.4.1).

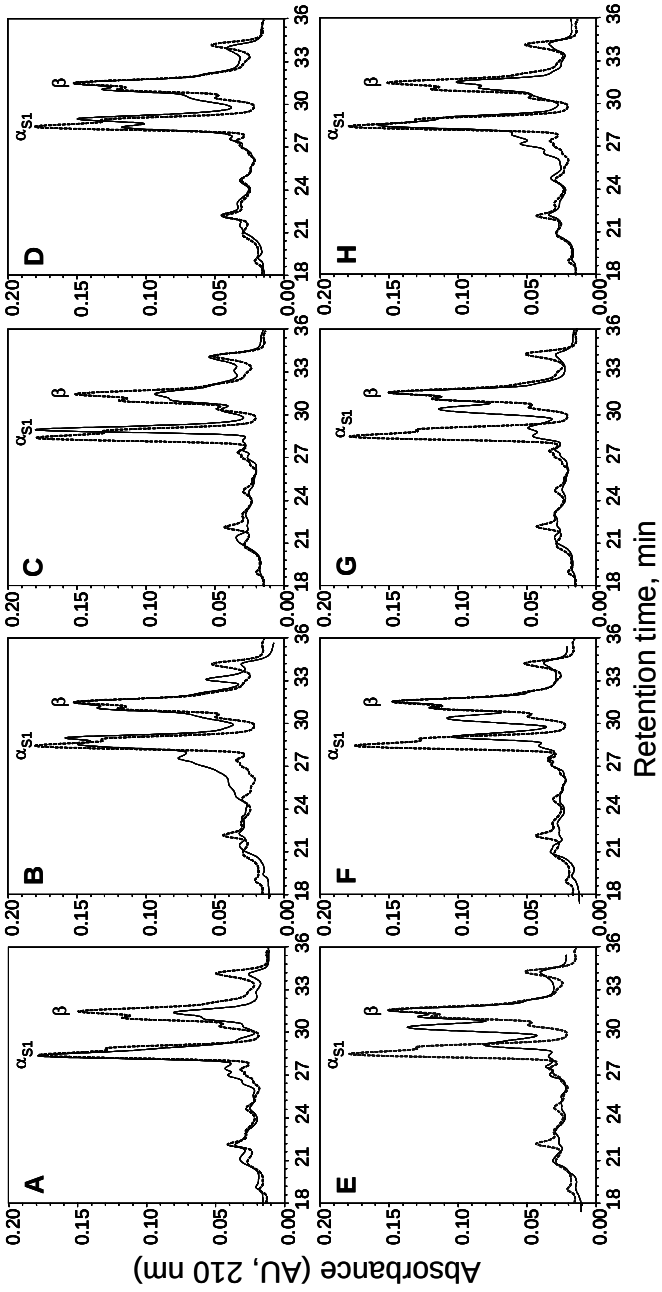

Figure 1.4.1. *RP–HPLC patterns of sodium caseinate digests after five hours of incubation with whole cells of LAB strains:* S. thermophilus *CNRZ 385 (A), 3207 (B),* L. acidophilus *ATCC 4356 (C), LA03 (D),* L. helveticus *MIMLh5 (E), Lh164 (F), ATCC 15009 (G), and* L. lactis *subsp.* lactis *GR5 (H). Solid lines, patterns after 5 h of incubation; dotted lines, patterns after 5 h of incubation with whole cells of* S. thermophilus *protease-negative strain DSM 20617. Letters* α_{S1} *and* β *indicate corresponding casein fractions.*

In accordance to the absence of CEP activity in *S. thermophilus* DSM 20617, chromatographic profile showed that sodium caseinate was not hydrolysed after treatment with this strain (Figure 1.4.1). Caseinate profiles in the presence and in the absence of *S. thermophilus* DSM 20617 cells were demonstrated to be identical (data not shown). On the contrary, in the case of sodium caseinate hydrolysis with *S. thermophilus* strain CNRZ 385, after five hours of proteolysis, we noticed the decrease of β-casein fraction and a slight digestion of α_{S1}-casein. The change in α_{S1}-casein fraction profile was demonstrated also with *S. thermophilus* strain 3207, whereas hydrolysis of β-casein was not appreciable.

In the case of *L. acidophilus* strain ATCC 4356, there is the shift in the location of the α_{S1}-casein peak from the "undigested casein" control that likely indicates the cleavage of a peptide from this casein fraction. This strain strongly digested β-casein, and the proteolytic activity against this protein was comparable to that observed for *S. thermophilus* strain CNRZ 385. Instead, LA03 had no effect on β-casein, but digested α_{S1} form. Similarly, but at a different degree, the three tested *L. helveticus* strains (MIMLh5, Lh164 and ATCC 15009), demonstrated to proteolyse only α_{S1} form. Finally, *L. lactis* subsp. *lactis* GR5 degraded β-casein and to a lesser extent α_{S1} form, similarly to that revealed for *S. thermophilus* CNRZ 385 strain.

As all analysed CEP-positive strains demonstrated proteolytic activity against sodium caseinate and showed the diverse hydrolytic ability against different casein fractions, this could determine diverse immunomodulatory activities of derived CHs.

1.4.2 Immunomodulatory activities of CHs

CHs, produced after digestion with proteinases of the eight CEP-positive bacterial strains, were tested for a potential immunomodulatory activity. In this regard, it has been evaluated the capability of hydrolysates to modulate the activation of transcription factor NF-κB in Caco-2 cells. These cells from human colorectal adenocarcinoma (Fogh et al., 1977), after about two weeks of differentiation, share the morphological and functional properties of mature intestinal cells, such as enterocytes or mucus cells, and therefore are valuable in vitro tools for studies related to intestinal cell function and immune response. NF-κB reporter system was chosen as this transcription factor regulates many physiological processes, including the innate- and adaptive-immune responses, cell death and inflammation (Gilmore, 2006; Perkins, 2007). NF-κB is a critical transcription factor that functions to enhance the transcription of many cytokines (including tumour necrosis factor (TNF)-α, IL-1, IL-6, and IL-8, which are thought to be important in the generation of acute inflammatory responses), growth factors, adhesion molecules, immunoreceptors, and acute-phase proteins. Activated NF-κB initiates both extracellular and intracellular regulatory events that result in autoregulation of the inflammatory cascade through positive and negative feedback loops (Blackwell & Christman, 1997).

On these bases, eight CHs, produced after digestion with LAB CEPs, were tested for their ability to modulate NF-κB activation in recombinant Caco-2 cells. Two of them, deriving from digestion with *L. helveticus* MIMLh5 and *L. acidophilus* ATCC 4356 CEPs, were found to significantly decrease the basal NF-κB activity (Figure 1.4.2), therefore they demonstrated immunomodulatory activity. These hydrolysates were selected for subsequent analysis.

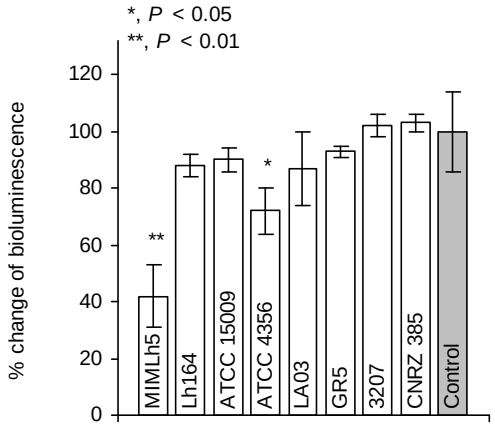

Figure 1.4.2. *Effects of whole CHs, produced after digestion with LAB strains on recombinant Caco-2 cells. Luciferase activity is expressed as percentual change in relative luminescence units, assuming the control as 100 %. Control, Caco-2 incubated with undigested sodium caseinate. The values are the means (± standard deviation) of at least three independent experiments conducted in duplicate. Different asterisks (*) indicate statistically significant differences compared to control.*

Several works in literature indicate the immunomodulatory activity induced by bovine milk fermentation products with strains of *L. helveticus*. The group of Matar published a series of papers, demonstrating immunomodulating effects of milks fermented by *L. helveticus* R389 and evidencing peptidic fractions, responsible for this activity (Matar et al., 1996; Matar et al., 2001; LeBlanc et al., 2002; LeBlanc et al., 2004). It was clearly demonstrated that the mentioned strain itself did not induce mucosal and tumoural immunity (Matar et al., 2001). Fujiwara and colleagues (Fujiwara et al., 1990) suggested that *L. helveticus* fermented milk products induced mitogenic activity. *L. helveticus* 5089-fermented milk has demonstrated immunomodulating effects on lymphocyte proliferation *in vitro* (Laffineur et al., 1996) and the ability to stimulate the phagocytic activity of pulmonary macrophages. However, both papers attribute immunomodulatory activity to casein-derived peptides and do not take in consideration the potential activity due to the presence of *L. helveticus* cell components in the fermented milks.

Since we have recently demonstrated that *L. helveticus* MIMLh5 strain by itself modulates the immune response (Guglielmetti et al., 2010b), it is paramount to avoid the presence of bacterial cell components in CHs. With this aim CH were ultrafiltered using a 3 kDa membrane and the permeate was tested for ability to modulate NF-κB activation in recombinant Caco-2 cells. In fact, the MIMLh5 hydrolysate after 3 kDa-ultrafiltration did not modulate NF-κB activation in recombinant Caco-2 cells, i.e. it lost immunomodulatory activity (Figure 1.4.3).

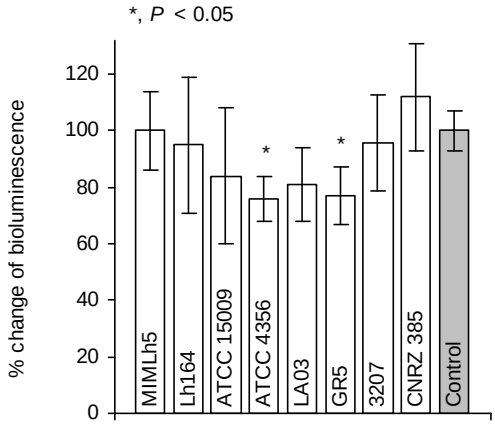

Figure 1.4.3. *Effects of fractionated (ultrafiltration 3 kDa) CHs, produced after digestion with LAB CEPs, on recombinant Caco-2 cells. Control, Caco-2 incubated with undigested and 3 kDa-ultrafiltered sodium caseinate. The values are the means (± standard deviation) of at least three independent experiments conducted in duplicate. Asterisks (*) indicate statistically significant differences compared to control.*

This result let us presume the possible presence in such "hydrolysates" of bacterial cell components that detach easily from cell surfaces during hydrolysate preparation. We proved the presumption that the observed difference was due to cell components, by incubating the cells under the same conditions, but in the absence of casein. Also in this case permeate of 3 kDa-ultrafiltration did not cause the significant decrease in NF-κB activation (Figure 1.4.4).

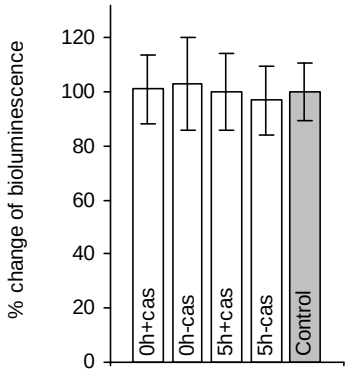

Figure 1.4.4. *Effects of fractionated (ultrafiltration 3 kDa) CHs, produced after 0 and 5 h digestion with* L. helveticus *MIMLh5 CEP, on recombinant Caco-2 cells in the presence (+cas) and in the absence (-cas) of sodium caseinate. 0h+cas, CH, prepared after 0 h digestion with bacterial proteinase; 0h-cas, hydrolysis reaction buffer, incubated 0 h with bacterial cells; 5h+cas, CH, prepared after 5 h of sodium caseinate contact with bacterial cells; 5h-cas, hydrolysis reaction buffer, incubated 5 h with bacterial cells. Control, Caco-2 incubated with undigested and 3 kDa-ultrafiltered sodium caseinate. The values are the means (± standard deviation) of three independent experiments conducted in duplicate.*

Secondly, we incubated "CH", produced after 0 h digestion with *L. helveticus* MIMLh5 proteinase, i.e. the non-hydrolysed casein after the short contact with bacterium. In this instance the non-fractionated sample determined a significant decrease of the basal NF-κB activity (Figure 1.4.5), in contrast to the observation that non-fractionated caseinate sample by itself does not modulate the NF-κB activation in Caco-2 reporter cell line.

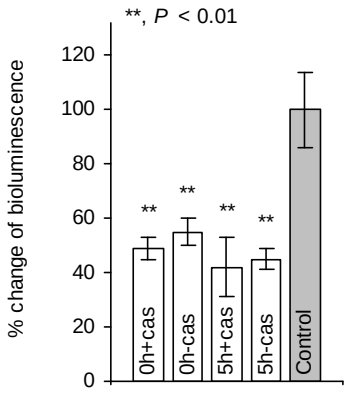

Figure 1.4.5. *Effects of non-fractionated CHs, produced after digestion (0 h and 5 h) with* L. helveticus *MIMLh5 CEP, on recombinant Caco-2 cells in the presence (+cas) and in the absence (-cas) of sodium caseinate. 0h+cas, CH, prepared after 0 h digestion with bacterial proteinase; 0h-cas, hydrolysis reaction buffer, incubated 0 h with bacterial cells; 5h+cas, CH, prepared after 5 h of sodium caseinate contact with bacterial cells; 5h-cas, hydrolysis reaction buffer, incubated 5 h with bacterial cells. Control, Caco-2 incubated with undigested sodium caseinate. The values are the means (± standard deviation) of three independent experiments conducted in duplicate. Asterisks (*) indicate statistically significant differences compared to control.*

These two results suggest the origin of immunomodulatory activity deriving from the tested bacterium and let us argue the absence of NF-κB activating peptides in CHs, produced by proteolytic activity of MIMLh5. In general, these results underline the importance to consider bacterial components of the > 3kDa size class when evaluating the immunomodulatory activity of non-fractionated CHs.

Subsequently, we continued our investigation with the other immunologically active CH, produced after digestion with CEP of *L. acidophilus* ATCC 4356.

Scarce data about immunomodulatory activity of *L. acidophilus* strains are present in the scientific literature. In one case the origin of this activity has been clearly demonstrated by Konstantinov et al. (2008) who showed that the immunomodulatory activity of *L. acidophilus* is determined by its S-layer. To our knowledge, the data about *L. acidophilus* ATCC 4356 immunomodulatory activity are absent. Analogously, we tested the immunomodulatory activity of the 3 kDa-ultrafiltered CH, produced after digestion with the CEP of *L. acidophilus* ATCC 4356. Contrary to CH produced after digestion with CEP of *L. helveticus* MIMLh5, the 3 kDa-ultrafiltered CH produced by *L. acidophilus* ATCC 4356 retains immunomodulatory activity demonstrating NF-κB activation in recombinant Caco-2 cell monolayers (Figure 1.4.3 and Figure 1.4.6A).

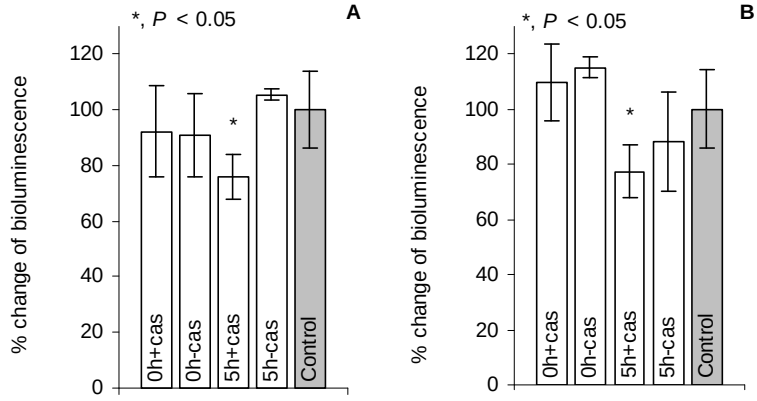

Figure 1.4.6. *Effects of fractionated (ultrafiltration 3 kDa) CHs, produced after digestion (0 h and 5 h) with* L. acidophilus *ATCC 4356 (A) and* L. lactis *subsp.* lactis *GR5 (B) CEPs, on recombinant Caco-2 cells in the presence (+cas) and in the absence (-cas) of sodium caseinate. 0h+cas, CH, prepared after 0 h digestion with bacterial CEP; 0h-cas, hydrolysis reaction buffer, incubated 0 h with bacterial cells; 5h+cas, CH, prepared after 5 h of sodium caseinate contact with bacterial cells; 5h-cas, hydrolysis reaction buffer, incubated 5 h with bacterial cells. Control, Caco-2 incubated with undigested and 3 kDa-ultrafiltered sodium caseinate. The values are the means (± standard deviation) of at least three independent experiments conducted in duplicate. An asterisk (*) indicates statistically significant differences compared to control.*

It can be supposed that non-fractionated CHs contain peptides that enhance or suppress NF-κB activation and, therefore, synergistic action of these peptides does not give immunomodulatory response. For this reason, other six CHs (that intact did not modulate NF-κB activation in recombinant Caco-2 cells), produced after digestion with CEP of *L. helveticus* Lh164 and ATCC 15009, *L. acidophilus* LA03, *L. lactis* subsp. *lactis* GR5, *S. thermophilus* 3207 and CNRZ 385, were ultrafiltered (3 kDa) and tested for immunomodulation. Interestingly, 3 kDa-fraction of CH produced by *L. lactis* subsp. *lactis* GR5 strain significantly decreased the basal NF-κB activity in Caco-2 reporter cell monolayers (Figure 1.4.3). To confirm or reject the possible presence of bacterial cell components in this fractionated CH, the GR5 strain was also

incubated for 0 and 5 h in the presence and in the absence of sodium caseinate. The derived hydrolysates were ultrafiltered through 3 kDa-membrane and tested for immunomodulatory activity. The abolishment of NF-κB activation, obtained with hydrolysates, produced after 5 h of digestion with CEP of *L. lactis* subsp. *lactis* GR5, was confirmed (Figure 1.4.6B).

Further, the anti-inflammatory activity of all 3 kDa-fractionated CHs was tested. For this reason, recombinant Caco-2 cells were co-stimulated with 3 kDa-fractionated CHs and IL-1β, a prototypical pro-inflammatory cytokine that plays a central role in the inflammation amplification cascade. The addition of 2 ng mL^{-1} of IL-1β to Caco-2 culture medium caused nearly a twofold increase of NF-κB activity after 4 h incubation. In this experiment we also used a synthetic anti-inflammatory tripeptide KPV (Primm, Milan, Italy) (Dalmasso et al., 2008) as a positive control. After stimulation by IL-1β, tripeptide KPV significantly reduced the NF-κB activation, while the control hydrolysate (intact sodium caseinate, ultrafiltered through 3 kDa membrane) did not have any significant effect (Figure 1.4.7).

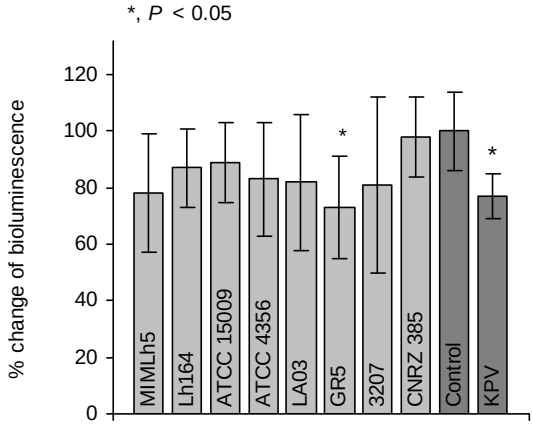

Figure 1.4.7. *Effects of fractionated (ultrafiltration 3 kDa) CHs, produced after digestion with LAB CEPs, on recombinant Caco-2 cells in the presence of co-stimulation with IL-1β. KPV, a synthetic tripeptide KPV, 10 nM. The values are the means (± standard deviation) of at least three independent experiments conducted in duplicate. An asterisk (*) indicates statistically significant differences compared to control.*

Finally, we observed that the 3 kDa-fraction of CH produced by *L. lactis* subsp. *lactis* GR5 strain significantly decreased the basal NF-κB activity in the presence of IL-1β thus showing anti-inflammatory activity in recombinant Caco-2 cell layers (Figure 1.4.7 and Figure 1.4.8).

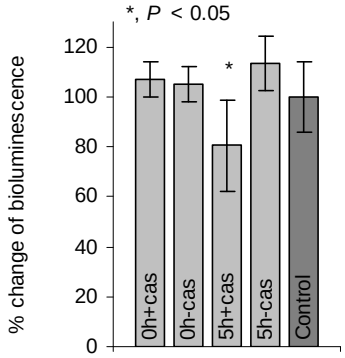

Figure 1.4.8. *Effects of fractionated (ultrafiltration 3 kDa) CHs, produced after digestion with* L. lactis *subsp.* lactis *GR5 CEP on recombinant Caco-2 cells in the presence of co-stimulation with IL-1β in the presence (+cas) and in the absence (-cas) of sodium caseinate. The values are the means (± standard deviation) of at least three independent experiments conducted in duplicate. An asterisk (*) indicates statistically significant differences compared to control.*

1.5 CONCLUSIONS

According to the tasks described in the chapter 1.2, the following conclusions were drawn:

1. In this study different proteolytic specificity of CEPs on casein fractions was observed among the tested LAB strains.
2. The developed stable NF-κB reporting Caco-2 cell line demonstrated to be an accurate tool for evaluating the immunomodulatory activity of CHs.
3. This work established that 3 kDa-ultrafiltered CHs, produced after digestion with CEPs of *L. acidophilus* ATCC 4356 and *L. lactis* subsp. *lactis* GR5, significantly decreased the basal NF-κB activity in recombinant Caco-2 cell layers, thus demonstrating immunomodulatory activity. Both the strains digested β-casein mostly.

In the case of *L. helveticus* MIMLh5, the investigation proved that the immunomodulatory activity of obtained CH was not due to casein-derived peptides. This activity was strong and comparable to the one exerted by bacteria themselves, as demonstrated by Guglielmetti et al. (2010b). Indeed, the surface layer (S-layer) protein of *L. helveticus* MIMLh5 was identified as responsible for the immunomodulation (Taverniti et al., manuscript in preparation). For this reason, the second part of the research was focussed on the development of S-layer protein-specific binders, a new powerful tool for the study of this immunologically active protein and for its identification in milk-derived products containing *L. helveticus* as a starter or non starter LAB.

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2 *LACTOBACILLUS HELVETICUS* MIMLh5 S-LAYER PROTEIN-SPECIFIC BINDERS FROM ANTIBODY LIBRARIES

2.1 STATE OF THE ART

Lactobacilli are rod-shaped Gram-positive non-spore-forming bacteria characterised by the formation of lactic acid as a sole or main end product of their sugar fermentation. Particularly, the genus *Lactobacillus* is the largest included in LAB with over hundred species recognised at present. It is very heterogeneous containing species with substantial differences in their phenotypic, biochemical, physiological and genotypic characteristics.

L. helveticus is a thermophilic homofermentative microorganism. Several strains are claimed to be health-benefiting to their host (Tuohy et al., 2003; Johnson-Henry et al., 2007; Guglielmetti et al., 2010) and are therefore currently used as probiotic supplements in several commercial products [e.g. "Evolus" (Valio, Finland), "Ferzym plus" (Specchiasol, Italy), "A'Biotica" (Institut Rosell, Canada), "All-Flora" (New Chapter, USA)]. The species demonstrates high proteolytic activity, which is favourable in some industrial processes, for instance highly contributing to cheese ripening. In fact, *L. helveticus* has an important role in the production of American Swiss, Emmental, Cheddar, Parmesan, Pecorino Romano, Provolone, Grana Padano cheeses (Lortal, 2004). Due to the long history of use in food fermentations and in the food industry as well as lack of pathogenicity, *L. helveticus* is a generally recognised as safe (GRAS) organism, which has received the Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA, 2004).

2.1.1 S-layer proteins of *Lactobacillus* spp.

Surface (S-) layers are crystalline arrays of proteinaceous subunits present as the outermost component of cell wall in several species of the genus *Lactobacillus*, as well as in many other bacteria and Archaea (Sára & Sleytr, 2000). S-layer is composed from one single protein or glycoprotein, forming crystalline structure of 5–15 nm with identical pores, which have diameter of 2–8 nm (Sleytr, 1997). The pores comprise up to 70 % of the lattice surface area (Sleytr & Beveridge, 1999). S-layer proteins have the ability to self-assemble into monomolecular crystalline arrays. The S-layer lattice can exhibit oblique (p1, p2), square (p4) or hexagonal (p3, p6) symmetry. From the *Lactobacillus* S-layer proteins, only oblique and hexagonal lattice types have been characterised (Figure 2.1.1), but in most cases, only a periodic structure, without further characterisation, has been observed using electron microscopy analysis. Problems related to electron microscopy of *Lactobacillus* S-layers have been reported, and these problems may be one of the reasons why the lattice type and lattice constants have not been determined for most of the S-layer proteins of these bacteria (Åvall-Jääskeläinen & Palva, 2005).

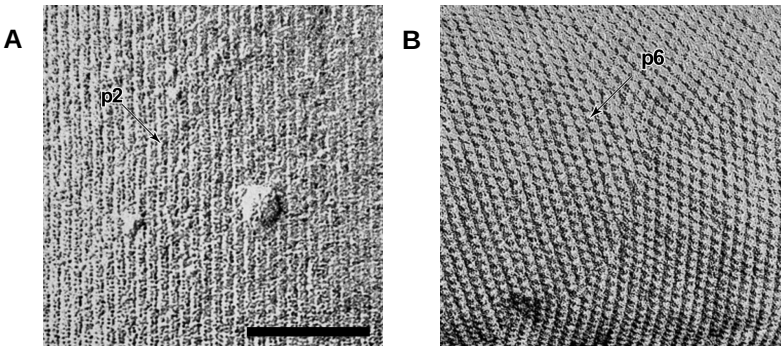

Figure 2.1.1. *Scanning electron micrograph of a freeze-etched preparation of a bacterial cell with an S-layer with oblique (p2; A) and hexagonal (p6; B) lattice symmetry. Scale bar = 100 nm. Adapted from Sleytr & Beveridge (1999).*

S-layer proteins are normally the most abundant bacterial proteins, constituting even up to 15 % of total bacterial protein content (Åvall-Jääskeläinen & Palva, 2005). In the case of *L. helveticus*, it forms around 45 % of cell wall dry weight (Messner et al., 1997).

Most of the S-layer proteins of the genus *Lactobacillus* appear to be non-glycosylated, and up to date all known *L. helveticus* S-layer proteins are known not to have this modification. The S-layer monomers are non-covalently linked to each other as well as to the supporting cell wall.

The overall lack of identity between the S-layer proteins of different species is a common characteristic of all bacterial S-layer proteins. Lactobacilli make no exception in this respect (Åvall-Jääskeläinen & Palva, 2005; Gatti et al., 2005). The highest similarity can be found in the C-terminal region. The N-terminal regions are more variable except the signal peptides (N-terminal secretion signals) that share a high level of sequence similarity.

Typical characteristics of *Lactobacillus* spp. S-layer proteins, distinguishing them from other S-layer proteins, is small size, ranging from 25 to 71 kDa and thus being among the smallest known for the S-layer proteins, which can be up to 200 kDa (Åvall-Jääskeläinen & Palva, 2005). S-layer proteins of lactobacilli have high content of positively charged residues, which makes them highly basic proteins: predicted isoelectric point (pI) values range from 9.35 to 10.4 (Mobili et al., 2010). A characteristic feature of *Lactobacillus* spp. S-layer proteins is a high percentage (23–33 %) of amino acid residues with hydroxyl groups and a high content of hydrophobic amino acid residues, ranging from 31.9 % to 38.7 %, which is a typical feature for all S-layer proteins (Åvall-Jääskeläinen & Palva, 2005).

The functional roles of *Lactobacillus* spp. S-layers are yet poorly characterised. It was demonstrated that it can be involved in mediating adhesion to different host surfaces, even if this is not the case for all the microorganisms that have this protein (Sára & Sleytr, 2000). It was demonstrated that S-layers of *L. helveticus* and *L. acidophilus* can participate in the interaction between bacterium and its host (Johnson-Henry et al., 2007; Konstantinov et al., 2008; Beganović et al., 2011). Konstantinov and colleagues demonstrated the ability of the S-layer protein of *L. acidophilus* NCFM to interact with human's immune system, determining the modulation of dendritic cell response and the activation of T-helpers (Konstantinov et al., 2008). It is worth noting that the S-layer of *L. acidophilus* NCFM is highly similar to that of *L. helveticus*. Beganović and collaborators found that the S-layer protein is involved in the autoaggregation of *L. helveticus* M92 cells and coaggregation of *L. helveticus* M92 with *Salmonella enterica* serovar Typhimurium FP1. Moreover, it enhances the immune protection by reducing the infection by *S. enterica* serovar Typhimurium FP1 *in vivo* (Beganović et al., 2011).

A general functional principle for all S-layers has not been determined and probably does not exist, due to the widespread occurrence of S-layer-possessing organisms and their differences in the overall cell surface structure. Due to their highly periodic and regularly arranged porous ultrastructure, S-layers possess great potential for various applications in biotechnology, nanotechnology, nanobiotechnology and biomedical applications (Sára & Sleytr, 2000; Sleytr et al., 2003; Åvall-Jääskeläinen & Palva, 2005; Pum et al., 2006; Schuster & Sleytr, 2009; Hollmann et al., 2010).

2.1.2 Binders from phage antibody libraries

There are two methods for generating of monoclonal antibodies. The first one comprehends mice immunisation followed by hybridoma technology. In this technology hybrid cell lines (called hybridomas) are formed by fusing a specific antibody-producing B cell with a myeloma (B cell cancer) cell that is selected for its ability to grow in tissue culture and for an absence of

antibody chain synthesis. The production of monoclonal antibodies was invented by Cesar Milstein and Georges J. F. Köhler in 1975.

Over the past twenty five years, advances in molecular biology have allowed to use the bacterium *Escherichia coli* for production of recombinant binders (antibodies) employing the phage display technology.

2.1.2.1 Phage display

Definition. In phage display, a foreign peptide, protein or antibody fragment is expressed on the phage surface due to the new genetic material inserted into a phage genome, fused to a capsid protein-encoding gene. The first report of phages displaying foreign peptides on their surface was published in 1985 (Smith, 1985).

Antibody formats. Usually only a fragment of the entire antibody is being expressed due to the size limitations for protein production in bacteria. However, this antigen-recognising part is enough for the complete functionality. Antibody fragments most often used in phage display are either of single-chain variable fragment (scFv) or Fab format (Figure 2.1.2).

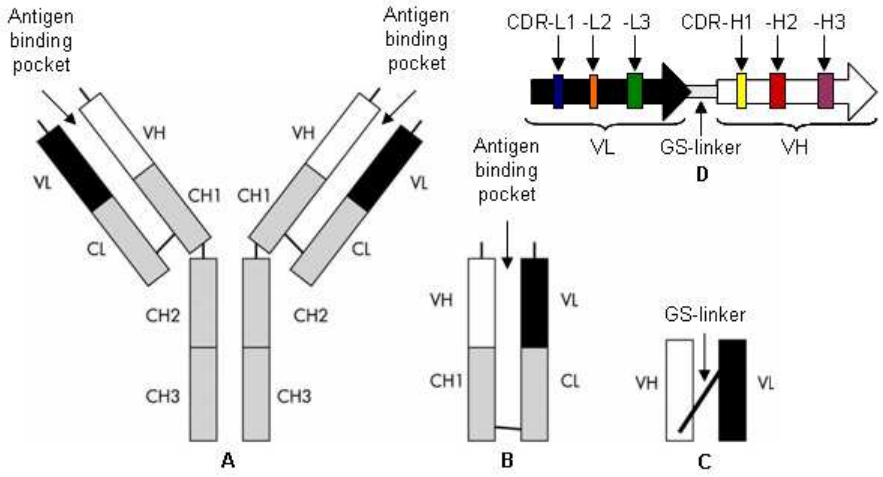

Figure 2.1.2. *Schematic antibody structure and derived fragments. (A) Schematic structure of an immunoglobulin molecule, (B) Fab and (C) scFv antibody fragments. Immunoglobulin consists of two identical heavy and light chains composed of variable and constant domains. VH, variable region of heavy chain; VL, variable region of light chain; CH, constant region of heavy chain; CL, constant region of light chain. The antigen-binding site is located at the tip of the variable domains. (D) The schematic structure of a scFv fragment gene. A scFv fragment consists of a VL and VH domains, which are linked through a flexible glycine-serine linker. Hypervariable complementarity-determining regions (CDR) that form the antigen-binding surface in a folded protein are indicated: CDR-L1, CDR-L2, CDR-L3, CDR-H1, CDR-H2, CDR-H3. Adapted from Smith et al. (2004) and Brockmann (2010).*

Display systems. Four kinds of display system have been developed so far, namely, phage lambda, T4 phage, T7 phage and filamentous phage, the filamentous M13 phage being the most widely distributed (Kehoe & Kay, 2005). In M13 filamentous phage display, several coat proteins have been used as targets for the fusion of foreign peptides. The first and most commonly used is the p3 coat protein (Smith, 1985), which is present at three to five copies in M13 (Figure 2.1.3). The major coat protein p8 (2670 copies per phage particle) is also used (Benhar, 2001).

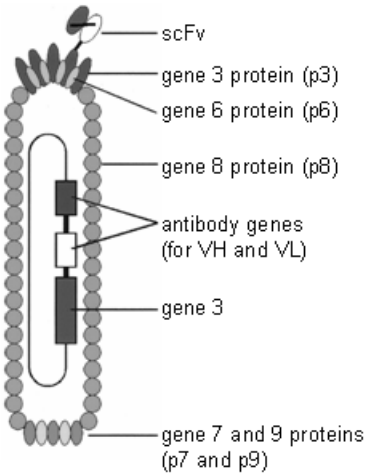

Figure 2.1.3. *Schematic structure of filamentous M13 phage and the principle of monovalent phage display. Adapted from Smith et al. (2005).*

Phage display formats. Phage display systems can be grouped into two classes on the basis of the vector system used for the production of phages (Paschke, 2006). (1) True phage vectors are directly derived from the genome of filamentous phage (M13, f1, or fd) and encode all the proteins needed for the replication and assembly of the filamentous phage. Early phage display formats involved the peptide, protein or antibody fragment-encoding gene fusion to the phage capsid protein-encoding gene. In this case, they were displayed in a multivalent format, since all copies of the coat protein are translated as fusion proteins (Carmen & Jermutus, 2002). However, it was not possible to display larger polypeptides or proteins without affecting the function of capsid protein or decreasing of phage infectivity. These problems are overcome by the use of phagemids. The second group of phage display systems utilises (2) phagemid vectors, which produce the fusion coat protein. Phagemids are plasmids (approximately 4.6 kilobases) which encode a signal sequence, the phage coat protein and an antibiotic resistance marker. The antibody fragment is cloned upstream (for N-fusions) or downstream (for C-fusions) of the capsid protein sequence and expression is controlled by the use of a promoter such as lacZ. The relatively small size of these vectors means that they have higher transformation efficiencies than phage vectors, hence facilitating the construction of large libraries of peptide or antibody fragments. The incorporation of an amber stop codon between the displayed protein and the phage coat protein permits fusion protein expression in suppressor strains of *E. coli* such as XL1-Blue. Non-suppressor strains, such as HB2151, will not incorporate a glutamine at the amber codon, thereby resulting in production of only the antibody moiety. A phagemid cannot produce infective phage particles alone. A helper phage (such as VCSM13; for monovalent display) or a hyperphage (a helper phage with a deletion in the p3 gene, but with wild type p3 phenotype, thus capable of infecting F^+ *E. coli* cells with high efficiency (for polyvalent display, Broders et al., 2003) is required. The helper phage provides the genes which are essential for phage replication and assembly. Cells already containing the phagemid vector are superinfected with the helper phage. Glucose in the growth media represses the lacZ promoter, preventing expression of fusion, which would inhibit superinfection. Once the helper phage genome is incorporated into the cell, the glucose is removed and phage production starts. In addition, lacZ promoter is being induced with IPTG.

Advantages of phage display technology. (1) Fast. One of the major advantages of phage display technology of antibody fragments compared with standard hybridoma technology is that the generation of specific fragment can be performed within a couple of weeks (Carmen &

Jermutus, 2002). As this method is based on microbial systems, selection and screening are more amenable to automation than earlier hybridoma-based approaches. This provides the potential for high-throughput generation of binders (Babel et al., 2011). (2) The immediate availability of the antibody gene provides much additional value relative to antibodies obtained by immunising animals. By permitting control over selection and screening conditions, display technologies allow (3) the generation of antibodies against defined antigen conformations or epitopes. Because the gene encoding the antibody is cloned at the same time as the antibody is selected, simple subcloning steps after *in vitro* display permit (4) the creation of constructs with added functionalities: fusion to enzymes or biotin, multimerisation. In addition, (5) antibody specificities can be broadened or narrowed by appropriate selection and screening. Antibody generation by phage display overcomes immunological tolerance, allowing the selection of (6) affinity reagents that recognise highly conserved targets such as histones, haemoglobins and post-translational modifications. On the other hand, *in vitro* display technologies allow (7) the generation of antibodies, which recognise antigens that are neither immunogenic nor highly conserved (e.g. small molecules and chemical modifications). By *in vitro* methods it is possible to select (8) antibodies against pathogens and toxins, that are not obtainable by traditional immunisation techniques. Unlike antibodies generated by immunisation, the *in vitro* selected ones (especially those of human origin) have the potential to be used therapeutically as molecules with improved tolerance by the patients.

The disadvantage of phage display selection of antibodies is that quite often antibodies with low affinities for the target are obtained. However, it turns to the major advantage of *in vitro* methods: once obtained it is possible to further improve the antibodies' affinity, specificity, expression or stability. The improvement of antibody affinity even to femtomolar range became possible (the affinities of antibodies obtained by immunisation are limited to ~100 pM by the physiological mechanism of B-cell activation) (Bradbury et al., 2011).

In their recent paper Andrew Bradbury and colleagues (Bradbury et al., 2011) raise a question: why antibodies selected by *in vitro* methods (even if being so powerful tools) are not widely perceived as valuable research reagents? Their answer is that difficult patent situation exists, which resulted in restriction of this technology to the high-margin therapeutic markets for commercial use. The authors note that in this regard hybridoma technology was never patented, and achieved relatively wide acceptance within a short period. They conclude that the situation for some of the core phage display patents is now changing rapidly, as most platform patents have either expired or will do so over the next few years.

2.1.2.2 Antibody libraries

Generally, three types of antibody libraries can be distinguished: immunised, naïve and synthetic (Hoogenboom & Chames, 2000; Carmen & Jermutus, 2002).The primary difference between these libraries is their origin. Immunised libraries are mainly derived from the immune repertoire of immunised animals, whereas most naïve and synthetic libraries are of human origin.

Immunised libraries are created to obtain antibodies specific for a certain antigen. They are assembled from rearranged variable domain genes isolated from immunised donors (Clackson et al., 1991).

Naïve libraries have been derived from germline immunoglobulin sequences (Söderlind et al., 2000). Construction of naïve libraries involves relatively straightforward molecular biology techniques such as reverse transcription of mRNA, followed by polymerase chain reaction (PCR) with germline-specific primers to amplify the V_H and V_L gene segments from the cDNA template, and restriction-based cloning to incorporate the rearranged antibody segments into an

appropriate phagemid display vector. Finally, the vectors are transformed into *E. coli* cells to generate the antibody repertoire.

Synthetic libraries are grafted from a single synthesised antibody molecule. Construction of synthetic libraries involves rearranging V_H and V_L gene segments *in vitro* and randomising the complementarity determining regions (CDRs) by PCR and randomised oligonucleotide primers, introducing varying loop lengths (Knappik et al., 2000; Brockmann et al., 2011).

2.1.2.3 Selection of binders from phage displayed libraries

Panning is the process for the selection and isolation of specific antibodies from antibody libraries by their binding activity. In principle, it involves the selection of binders/antibodies on the basis of their affinity. The isolation of a desired antibody generally involves repeated rounds of panning, with each successive round resulting in the enrichment of the desired antibody. Each round of antibody selection can be divided into exposing of phage to immobilised target, removal of nonspecific phage, and the elution and amplification of phage antibodies for the next round (Figure 2.1.4).

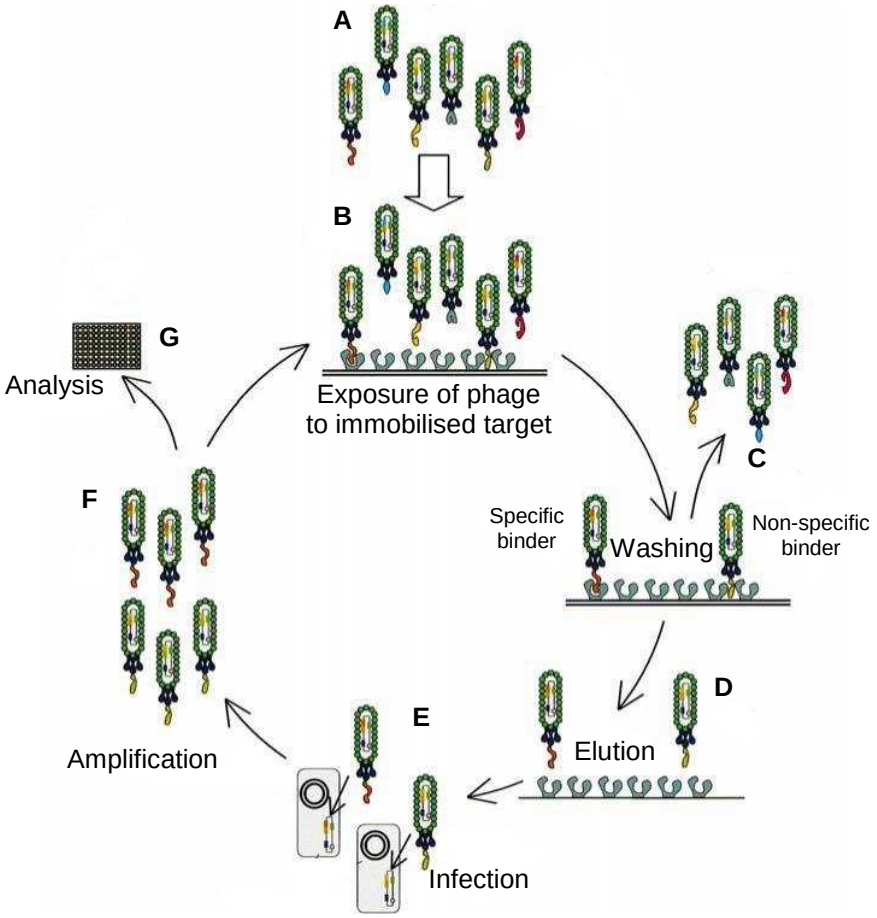

Figure 2.1.4. *The phage display cycle.*

Figure 2.1.4. (continued) *(A) A library of variant DNA sequences encoding peptides or proteins is created and cloned into phage or phagemid genomes as fusions to a coat protein gene. (B) The phage library displaying variant peptides or proteins is exposed to target molecules and phage with appropriate specificity are captured. (C) Non binding phage are washed off – although some non-specific binding may also occur. (D) Bound phages are eluted by conditions that disrupt the interaction between the displayed peptide or protein and the target. (E) Eluted phage are infected into host bacterial cells and thereby amplified. (G) This amplified phage population is in effect a secondary library that is greatly enriched in phage displaying peptides or proteins that bind to the target. If the bio-panning steps (B) to (E) are repeated the phage populated becomes less and less diverse as the population becomes more and more enriched in the limited number of variants with binding capacity. (G) After several (usually three to five) rounds of bio-panning monoclonal phage populations may be selected and analysed individually. Adapted from Willats (2002).*

2.2 AIMS OF THE STUDY

Recently, the potential health-promoting properties at the pharyngeal level of the dairy bacterium *L. helveticus* MIMLh5 were evaluated *in vitro* and *ex vivo*. The strain was proposed as a potential pharyngeal probiotic because of its ability to adhere to human epithelial cell lines and to efficiently antagonise group A streptococci on these cells (Guglielmetti et al., 2010). Furthermore, *L. helveticus* MIMLh5 appears to be a promising probable modulator of the immune system, which is able to reduce NF-κB activation, to influence cytokine secretion at the epithelial level, and potentially to skew the immune system toward a Th1 response.

It was already demonstrated that the probiotic activity of *L. helveticus* can be due to the presence of the S-layer protein (the case of strain M92; Beganović et al., 2011). It was suggested that *L. acidophilus* NCFM is the first probiotic bacterium identified that is functionally involved in the modulation of dendritic cells and T cells functions and this immunomodulatory activity is due to its S-layer protein (Konstantinov et al., 2008). It is worth noting that the S-layer protein of *L. acidophilus* NCFM is highly similar to that of *L. helveticus*. A new study is now in progress to identify the bacterial cell components involved in the immunomodulatory properties of *L. helveticus* MIMLh5 (Taverniti et al., manuscript in preparation).

For the reasons mentioned above we were interested to know whether the S-layer protein of *L. helveticus* MIMLh5, the potentially immunologically important protein, is present in food products, namely in Grana Padano cheese, whose natural whey starter (NWS) it was isolated from.

Therefore, the aim of the present study was to determine the presence of the *L. helveticus* MIMLh5 S-layer protein in Grana Padano cheese.

Most specifically the tasks were:

1. To set up an efficient extraction protocol to obtain pure extracts of *L. helveticus* MIMLh5 S-layer protein.
2. To determine the mass and the sequence of the *L. helveticus* MIMLh5 S-layer protein.
3. To select the *L. helveticus* MIMLh5 S-layer protein-specific binders, by employing the antibody phage display technology.
4. To set up an assay for the detection of *L. helveticus* MIMLh5 S-layer protein in Grana Padano cheese.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains and culture conditions

Table 2.3.1 lists the LAB strains used in this study. *L. helveticus* strains were grown at 42 °C in MRS broth (Sigma–Aldrich, Milan, Italy) supplemented with 1 % of Tween 80 (Sigma–Aldrich). *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus* were cultivated in the same medium at 37 °C. *Escherichia coli* XL1-Blue [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]] (Stratagene, Santa Clara, CA, USA) was grown in Luria–Bertani (LB) broth or SB medium at 37 °C, if not indicated diversely.

Table 2.3.1. *LAB strains included in the study.*

Species	Strain	Isolation source	Reference
L. helveticus	MIMLh5[a]	Grana Padano cheese NWS	Guglielmetti et al., 2010
	ATCC 15009^{T}	Emmental (Swiss) cheese	Orla-Jensen, 1919
	SLh02[b]	Fermented milk product	Unpublished
L. acidophilus	ATCC 4356^{T}	Human	Efthymiou & Hansen, 1962
	NCFM[c]	Human intestine	Sanders & Klaenhammer, 2001
L. delbrueckii subsp. *bulgaricus*	ATCC 11842^{T}	Bulgarian yogurt	Orla-Jensen, 1919

[a] DiSTAM Industrial Microbiology Section Culture Collection, University of Milan (Milan, Italy).
[b] Isolated from "Narine" (Vitamax-E, Yerevan, Armenia), Sacco S.r.l. Culture Collection (Cadorago, Italy).
[c] Isolated from "Probactiol" (Metagenics, San Clemente, CA, USA).
[T] Type strains from American Type Culture Collection (Manassas, VA, USA).

2.3.2 Extraction and purification of S-layer protein from *L. helveticus* MIMLh5

Extraction of the S-layer protein from *L. helveticus* MIMLh5 was performed with LiCl as described previously (Lortal et al., 1992; Smit et al., 2001; Johnson-Henry et al., 2007; Agave BioSystems, 2011). Briefly, 500 mL of an overnight culture of MIMLh5 was harvested by centrifugation at 3000 g for 20 min at 4 °C and washed with 1 volume of cold sterile MilliQ water. The cell pellet was extracted with 0.1 volume of 1 M LiCl solution for 30 min at room temperature in the presence of Protease Inhibitor Cocktail (Sigma–Aldrich, St. Louis, MO, USA) with slight agitation and centrifuged. The cell pellet was then extracted with 0.1 volumes of 5 M LiCl solution for 1 h at room temperature in the presence of Protease Inhibitor Cocktail and centrifuged once more. The supernatant was filtered through a 0.2 μm filter and exhaustively dialysed for 36 h at 4 °C against distilled water using 12000 kDa cut-off membranes (Sigma–Aldrich), which were prepared for dialysis by boiling in 2 % of $NaHCO_3$ and 1 mM EDTA solution. Each time changing the water 0.001 % of Protease Inhibitor Cocktail was added. The dialysate was collected and centrifuged at 20000 g for 20 min at 4 °C. The supernatant was removed and the pellet resuspended in several millilitres of sterile MilliQ water, shelf frozen and freeze dried. Protein concentration was determined by using the Bradford microassay method using bovine serum albumin (BSA) as a standard. Protein purity

was determined by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and RP–HPLC analysis.

2.3.3 SDS–PAGE

S-layer protein and total bacterial lysates were resuspended in SDS–PAGE (Laemmli) sample buffer (31.25 mM Tris pH 6.8, 12.5 % glycerol, 1 % SDS, 0.005 % bromophenol, 2.5 % β-mercaptoethanol), boiled for 5 min, and separated on 10 % SDS–PAGE in TRIS–glycine–SDS buffer on Mini-PROTEAN 3 system (Bio-Rad Italia, Milan, Italy). Gels were stained with Coomassie brilliant blue G-250 (Sigma–Aldrich).

2.3.4 RP–HPLC of S-layer protein

Samples were analysed by RP–HPLC on Waters Alliance 2695 instrument (Waters, Vimodrone, Italy). Protein was separated at 40 °C on a PLRP-S column (2.1 mm i.d., 5 μm, 300 Å, Polymer Laboratories Ltd, Church Stretton, UK). The eluents used for the separation were: solvent A, 0.1 % TFA in MilliQ-treated water, and solvent B, 0.1 % TFA in acetonitrile. The linear elution gradient expressed as the solvent B proportion was as follows: 0 min, 25 %; 0 to 5 min, 25 %; 5 to 35 min, 25 to 55 %; 35 to 36 min, 55 to 95 %; 36 to 38 min, 95 %; 38 to 39 min, 95 to 25 % (run–to–run time, 40 min). Protein was eluted at a flow rate of 0.2 mL min^{-1} and monitored at 210 nm with Waters 2996 Photodiode Array Detector (Waters).

2.3.5 Evaluation of immunomodulatory activity of *L. helveticus* MIMLh5 S-layer protein

The immunomodulatory activity of *L. helveticus* MIMLh5 S-layer protein was determined according to the method described previously (chapter 1.3.7) with some modifications. Recombinant Caco-2 cells were cultured in 24-well plates in presence of 50 μg mL^{-1} zeocin until the confluence. Cell monolayers (approximately 3×10^5 cells $well^{-1}$) were carefully washed with PBS buffer (pH 7.4), and 0.5 mL of fresh EMEM medium, containing 100 mM of HEPES (pH 7.4) and 100 μg of the *L. helveticus* MIMLh5 S-layer protein, was added. Caco-2 cells were stimulated and NF-κB activation was determined as described above. All samples were analysed in duplicate in at least three independent experiments.

2.3.6 Determination of molecular mass of S-layer protein by mass spectrometry

For liquid chromatography–mass spectrometry (LC–MS) analysis, S-layer protein was resuspended in MilliQ water or dissolved in 8 M urea. Mass determination analysis was performed on Waters Acquity UPLC instrument (Waters, Vimodrone, Italy) connected to a Q-Tof *micro* mass spectrometer (Waters) equipped with an orthogonal electrospray source (Z-spray). Protein was separated at 40 °C on Acquity UPLC BEH300 C4 column (2.1×150 mm, 1.7 μm, Waters). The eluents used for the separation were: solvent A, 0.1 % formic acid in MilliQ-treated water, and solvent B, 0.1 % formic acid in acetonitrile. The linear elution gradient expressed as the solvent B proportion was as follows: 0 min, 25 %; 0 to 4.78 min, 25 %; 4.78 to 22.78 min, 25 to 55 %; 22.78 to 23.38 min, 55 to 95 %; 23.38 to 24.58 min, 95 %; 24.58 to 25.18 min, 95 to 25 % (run–to–run time, 27 min). Protein was eluted at a flow rate of 0.2 mL min^{-1} and monitored at 210 nm with Acquity UPLC Photodiode Array Extended λ Detector (Waters). Mass spectra acquisition was performed in positive ion mode. The optimised mass spectrometry conditions were: capillary voltage 3200 V, source temperature 100 °C, and cone voltage 45 V. The electrospray ionisation (ESI) gas was nitrogen. The Tof analyser collected data between m/z 500 and 2000. The acquired MS spectrum was analysed with the MassLynx 4.1 software (Waters). MS data were processed using *MaxEnt 1* algorithm.

2.3.7 Determination of S-layer coding gene sequence from *L. helveticus* MIMLh5 and its analysis

Determination of S-layer coding gene was performed by PCR using specific primers: SLY ex F, CTGCAACTGCTATGCCTGT and SLY ex R, ATACGCTTAGTACCATCGA on the DNA extracted from the isolate according the standard procedure (Sambrook et al., 2001). The PCR conditions were as follows: predenaturation at 94 °C for 2 min, 35 cycles at 94 °C for 45 s, 56 °C for 45 s, 72 °C for 1.5 min, and a single final extension at 72 °C for 7 min. All amplification reactions were performed in a MyCycler thermal cycler (Bio-Rad Italia, Milan, Italy). PCR products were purified using PCR Clean-Up Kit (MO BIO Laboratories, Carlsbad, CA, USA) and sequenced with the dideoxy chain–termination principle (Sanger et al., 1977) employing Applied Biosystems Big Dye Chemistry v3.1 (Applied Biosystems, CA, USA).

Homology searches of the databases were done with the *BLAST* service at the National Center for Biotechnology Information (Altschul et al., 1990). Sequence alignments were performed using *MEGA 5* (Tamura et al., 2011) and *Align X* module of *Vector NTI Advance™ 11* (Invitrogen, Finland).

The isoelectric point and the theoretical molecular weight of the protein were calculated with *Compute pI/Mw* tool from *ExPASy* (Gasteiger et al., 2005).

2.3.8 Coating procedure

S-layer protein was coated on microtiter wells by passive adsorption. Lyophilised S-layer protein was dissolved in 5 M LiCl solution in mQ water up to 50 μg mL^{-1} concentration or later diluted up to 10 μg mL^{-1} with 5 M LiCl. MaxiSorp™ and PolySorp™ microtitration plates in 12-strip well formats were from Nunc (Roskilde, Denmark). The normal coating procedure for S-layer protein, the preparation of the control plate, is briefly described below. S-layer protein was diluted in the coating buffer (100 mM TRIS–HCl, pH 9.0) to the final concentration 5.0 μg mL^{-1} (or 1.0 μg mL^{-1}). Then 200 μL of the coating solution was dispensed into each well, giving 1 μg (for pannings) and 0.2 μg (for immunoassays) S-layer protein per well. The plates were closed in a humidified box and incubated overnight at room temperature (approximately 25 °C). Then the plates were washed twice in a DELFIA Platewash (Perkin-Elmer Life Sciences, Turku, Finland) with DELFIA Wash Solution supplemented with Tween 20 (Merck, Hohenbrunn, Germany) to the final concentration 0.05 %. After washing, 250 μL of saturation solution (50 mM Tris–HCl, pH 7.0; 150 mM NaCl; 0.05 % NaN_3; 6 % D-sorbitol) with BSA (0.2 % bovine serum albumin fraction V powder; γ-globulin free, Sigma–Aldrich) or skim milk (1 %) was added per well. The plates were saturated overnight at room temperature (approximately 25 °C). The saturation solution was aspirated in a DELFIA Platewash and the plates were dried (25 °C, under the laminar hood) for 4 h. Finally the plates were packed with moisture adsorbent into an aluminium zip-bag and stored dry at 4 °C. The control plates were prepared coating microtiter wells only with saturation solution, containing BSA or skim milk.

2.3.9 Synthetic human antibody libraries, vectors and helper phage

Synthetic human phage displayed scFv protein and hapten MolBind libraries (total diversity, 1.40×10^{10}) were provided by the Department of Biotechnology, University of Turku (Turku, Finland) cloned into pEB32x phagemid. The libraries are based on a single human framework. The framework gene is composed of a single human $V_L$kappa and a single V_H chain sequence. Chains were diversified by Kunkel mutagenesis at positions in the antigen binding site (CDR loops) that make contact to antigens (proteins and haptens, respectively), and subjected to affinity maturation by V_L shuffling (Brockmann et al., 2011). The scFv fragments comprise a

single polypeptide with the V_H and V_L domains attached to one another by a flexible 20 amino acid long glycine–serine linker.

The vectors used are shown in Figure 2.3.1.

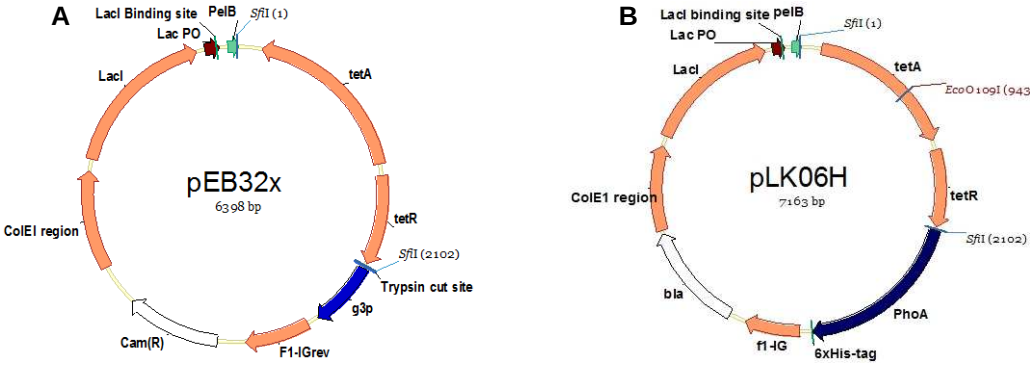

Figure 2.3.1. *Phagemid pEB32x (A) and expression vector pLK06H for 6×His-tagged bacterial alkaline phosphatase (PhoA) fusions (B).*

Phagemid vector pEB32x was used for monovalent phage display with helper phage VCS M13 (Stratagene, La Jolla, CA, USA). It conferred chloramphenicol resistance. Vector pLK06H (containing β-lactamase encoding gene (*bla*) for ampicillin resistance) was used for screening antibody clones. In this vector scFv was cloned through *Sfi*I restriction sites and expressed as fused to bacterial alkaline phosphatase (PhoA). His-tag from this vector allows affinity purification using Ni-NTA matrix. Both vectors had IPTG inducible Lac promoter. Protein was directed to periplasm of *E. coli* by pelB signal sequence. Vectors pEB32x and pLK06H are derivatives of pAK200 and pAK600, respectively (Krebber et al., 1997). They were kindly provided by the Department of Biotechnology, University of Turku, Finland. Primer pAKfor 5'-TGAAATACCTATTGCCTACG-3' was used for sequencing of scFv fragment from the vector pLK06H.

2.3.10 Panning on microtiter wells

Panning is the process for the selection and isolation of specific antibodies by their binding activity. S-layer coated microtiter wells were panned with a mixture 1 : 1 of pEB32x scFv hapten MolBind and pEB32x scFv protein MolBind phage libraries (Brockmann et al., 2011). This phage library mix (2.5×10^{12} cfu mL^{-1}, dilution in TBT-0.05) was first incubated in a well (200 µL $well^{-1}$) not coated with S-layer protein for 1 h at room temperature with slow shaking (Delfia® plateshake, low mode, Perkin-Elmer Life Sciences, Turku, Finland), upon which the unbound fraction was transferred to S-layer-coated wells. After 2 h (for the first panning) and 1 h (for the subsequent pannings) of incubation, the wells were washed 3 times (for the first panning) and 4 times (subsequent panning) with TBT-0.1, then 1 time with TBS-0.1 % Tween 20. The bound phages were then eluted with 200 µL of trypsin solution (60 µg mL^{-1} in TBS) for 15 min at room temperature and after neutralised with 1/10 volume of trypsin inhibitor (1 mg mL^{-1} in TBS).

The eluate was amplified by infection to *E. coli* XL1-Blue. The phages were purified by precipitation with PEG-8000 (16 % w/v)/NaCl (12 % w/v) on ice for 10 min and titrated using

LB agar plates with tetracycline (10 µg mL^{-1}) and chloramphenicol (25 µg mL^{-1}). The titrated phage was then diluted to 1×10^{11} cfu mL^{-1} in TBT-0.05 and applied as the input phage for subsequent pannings.

2.3.11 Screening for the binding specificity of phage antibodies to purified S-layer protein

To screen for binding specificity of phage library stocks (scFv-p3 fusions), they were diluted 1:10000 in Red Kaivogen Assay Buffer (Kaivogen, Turku, Finland) and analysed by DELFIA® time-resolved fluorescence immunoassay. In the assay, an antigen, the S-layer protein, was firstly bound to MaxiSorp™ and PolySorp™ microtitration wells, 0.2 µg $well^{-1}$, as indicated above. Then phage samples were incubated for 1 h in slight agitation (DELFIA® plateshake, low mode; Perkin Elmer Life Sciences, Turku, Finland) and after washing the plate Eu-N1-labelled anti-phage Mab (University of Turku, Turku, Finland) was bound. Time-resolved fluorescence signal of Eu^{3+} was measured with Victor 1420 multilabel counter (Perkin Elmer Life Sciences, Turku, Finland) after 10 min development with Delfia enhancement solution.

To screen for binding specificity of individual phage antibody clones, the enriched library was cloned into vector pLK06H (through *Sfi*I restriction sites), which expresses scFv as a fusion to PhoA and 6×His-tag. Individual clones were inoculated in SB medium (0.05 % glucose, 10 µg mL^{-1} tetracycline, 100 µg mL^{-1} ampicillin) onto a 96-well V-bottom culture plate (Corning Life Sciences, Pittston, USA) covered with a breathable sealing tape (Nunc, Roskilde, Denmark). Clones were grown for 4–6 h at 37 °C, 700 rpm, 70 % humidity. The cells induced with 100 µM IPTG and incubated overnight at 26 °C, 700 rpm. For **periplasmic extraction**, 1/5 volume of freshly prepared 5× lysis buffer (350 mM TRIS, pH 8.0, 10 mM EDTA, 10 mg mL^{-1} lysozyme) was added. Plates were incubated 10 min at 30 °C, 700 rpm and subsequently centrifuged 10 min at 4 °C, 3220 g. Supernatants were analysed by alkaline phosphatase (AP) chromogenic ELISA assay. In the assay, samples, diluted 1:10 with Red Kaivogen Assay Buffer were incubated for 1 h in the microtitration wells, coated with S-layer protein. After washing the plate 4×, substrate, para-nitrophenyl phosphate (pNPP) solution (50 mM TRIS, pH 9.0, 200 mM NaCl, 1 mM $MgCl_2$, 5 mM pNPP,) was added. Absorbance of p-nitrophenolate at 405 nm was measured with Victor 1420 multilabel counter (Perkin Elmer Life Sciences, Turku, Finland) after 1 h of colour development. A clone was considered to be positive if the specific signal and relative absorbance (A_{405}S-layer-coated well – A_{405}S-layer-uncoated well) were above 0.5 (A_{405}).

The test of specificity of the *L. helveticus* MIMLh5 S-layer protein-binding clones was based on phage binding to different S-layer containing LAB strains in suspension, and it was performed as indicated bellow.

2.3.12 Screening for the binding specificity of phage antibodies to S-layer protein-containing LAB strains

To screen for the MIMLh5 strain S-layer-specific phage antibodies, the MIMLh5 S-layer-positive scFv binder clones were inoculated in 5 mL of SB medium (0.05 % glucose, 10 µg mL^{-1} tetracycline, 100 µg mL^{-1} ampicillin) in culture tubes and cultivated at 37 °C with 300 rpm shaking to OD (600 nm) 0.8. The cultures were induced with 100 µM IPTG and grown further overnight at 26 °C with 250 rpm shaking. Cells were harvested by centrifugation 3220 g for 10 min at 4 °C. The pellet was resuspended in 1 mL (i.e. 5× concentrated) of modified Kaivogen Assay Buffer [mKAB, 50 mM TRIS–HCl, pH 7.5, 0.9 % NaCl, 0.01 % Tween-40, 0.5 % BSA (fraction V powder, Sigma–Aldrich, St. Louis, MO, USA)]. Cells were disrupted by sonication

and by two subsequent freeze (–70 °C) and thaw cycles. The lysate was centrifuged 15700 g for 5 min at 4 °C and the supernatant containing soluble anti-S-layer-scFv-PhoA binders was aliquoted and stored at –20 °C before AP chromogenic immunoassay in suspension. In the assay, lactobacilli cells were grown over night in MRS medium, harvested by centrifugation 3200 g, 5 min at 4 °C, resuspended in mKAB and "discharged" for 30 min at bacterial growth temperature. Then cell pellets were washed with mKAB and resuspended in the same buffer up to approximately 10^9 cells mL^{-1}. 100 µL of cell suspensions were aliquoted (approximately 10^8 cells/tube). The number of cells was selected empirically before by testing different cell concentrations (e.g. 10^8, 10^9 cells mL^{-1}). 100 µL of 5× diluted in mKAB lysates, containing anti-S-layer-scFv-PhoA binders, were added. LAB cell and scFv-PhoA binder suspensions were incubated for 1 h at room temperature in rotational agitation (13 rpm, Grant-bio PTR-30 rotator, Grant Instruments, Cambridge, UK). After washing 2× (the number of washes was selected empirically) with cold mKAB, a substrate, pNPP, solution was added and suspension was incubated at room temperature for 2 hours in rotational agitation (13 rpm). Cells were precipitated by centrifugation at 15700 g, 5 min, 4 °C and the absorbance of p-nitrophenolate in the supernatant was measured as described above. A clone was considered to be a positive binder to S-layer protein if the specific signal was above 3.0 or the relative absorbance (A_{405}MIMLh5 – A_{405}Lb11842) was above 1.0 (OD_{405}).

2.3.13 Preparation of anti-S-layer binder (anti-S-layer scFv-PhoA-6×His fusion) extracts for Western blotting

Anti-S-layer-scFv-PhoA-6×His fusion extracts were prepared under native conditions as indicated below. Briefly, cultures of 40 mL SB (10 µg mL^{-1} tetracycline, 100 µg mL^{-1} ampicillin, 0.05 % glucose) were inoculated with precultures to OD (600 nm) 0.05 and grown at 37 °C with 300 rpm shaking to OD (600 nm) 0.8. The cultures were induced for periplasmic expression of anti-S-layer-scFv-PhoA-6×His binders with 100 µM IPTG and grown further overnight at 26 °C with 250 rpm shaking. Cells were harvested by centrifugation 3220 g for 15 min at 4 °C. The pellet was resuspended in 1.2 mL of Lysis Buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) giving the final volume of approximately 1.5 mL. 2 mg mL^{-1} of lysozyme, 5 mM of $MgCl_2$, 112.5 U of DNase (Ambion, Applied Biosystems, Espoork, Finland), 1 mM PMSF were added. Suspensions were incubated 30 min at room temperature in rotational agitation and set on ice. Samples were further sonicated on ice and centrifuged 15700 g for 5 min at 4 °C on table top centrifuge. The supernatant, soluble fraction, was filtered through 0.2 µm ∅ syringe filter, pipetted to fresh tubes and stored at –20 °C.

2.3.14 Preparation of LAB total protein extracts for Western blotting

For preparation of total protein extracts, LAB cells were cultivated overnight in MRS medium, harvested by centrifugation 3220 g for 15 min at 4 °C. Cell pellet was washed, resuspended in cold PBS buffer, containing 1 mM PMSF and subjected to disruption by French Press E 1061 machine (Constant Systems, Daventry, UK) as indicated by manufacturer. Briefly, *Lactobacillus* spp. cells were resuspended in cold PBS buffer, containing 1 mM PMSF at a ratio 1 : 5 (wet weight of biomass vs. volume of resuspension buffer) and subjected to the pressure of 35000 psi for 3 times consecutively. Cell lysate was collected, aliquoted and stored at –70 °C before loading on polyacrylamide gel. For Western blotting analysis, 120 µg of total protein extract (per lane; concentration measured according A_{280}) was boiled for 5 min in SDS–PAGE (Laemmli) sample buffer (31.25 mM Tris pH 6.8, 12.5 % glycerol, 1 % SDS, 0.005 % bromophenol blue, 2.5 % β-mercaptoethanol) and separated on 10 % SDS–PAGE.

2.3.15 Detection of *L. helveticus* MIMLh5 S-layer protein by Western blotting

For denaturing conditions, proteins from SDS–PAGE gels were transferred to polyvinylidene fluoride (PVDF) membrane (Hybond-P, Amersham Biosciences, Buckinghamshire, UK) at a constant voltage of 15 V at room temperature for 15 min on a Trans-Blot® SD Semi-Dry Transfer Cell Module (Bio-Rad Laboratories, Richmond, CA, USA) in 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) electroblotting buffer system (10 mM CAPS, pH 11, 10 % methanol). For native conditions, *Lactobacillus* spp. total protein extracts were loaded as dots on the membrane and let to be absorbed passively. Membranes were blocked for at least 1 h in 5 % (w/v) non-fat dry milk in TBS (TRIS-buffered saline, pH 7.5) at room temperature. Further, blotting was done using anti-S-layer binder (anti-S-layer scFv-PhoA-6×His fusion) extracts in 1:200 dilution (in TBS with 0.05 % Tween20) as primary antibody (incubation overnight at 8 °C in rotational agitation) and horse radish peroxidase (HRP)-conjugated anti-His mouse monoclonal IgG1 (5Prime, VWR International, Helsinki, Finland) in 1:2000 dilution (in TBS with 0.05 % Tween20) as secondary antibody (incubation for 1 h at room temperature in rotational agitation). The membrane was visualised (chromogenic detection) with CN/DAB Substrate Kit (Thermo Scientific, Rockford, USA) with two consecutive substrate exposures.

2.3.16 Preparation of Grana Padano cheese samples for Western blotting

Samples from different stages of Grana Padano cheese maturation are listed in Table 2.3.2. They were kindly supplied by the Consorzio Tutela Grana Padano with the following specifications: dates of production and sample collection, registration number, indicating the manufacturer and the production site.

Table 2.3.2. *Grana Padano samples used for the study.*

Sample	Age	Production date	Sampling date	Registration No.
NWS	N/A		01.06.2011.	MN437
Curd	48 hours	01.06.2011.	01.06.2011.	MN437
Grana Padano "cheese"*	2.0 months	03.2011.	30.05.2011.	PC540
	3.5 months	15.02.2011.	31.05.2011.	MN401
	3.6 months	13.02.2011.	01.06.2011.	MN437
	4.0 months	01.2011.	30.05.2011.	PC540
	5.0 months	11.2010.	27.04.2011.	PD711
	5.0 months	12.2010.	25.05.2011.	MN410
	5.0 months	12.2010.	25.05.2011.	VI610

N/A, not applicable

* GP can be named as cheese only from the 9th month of ripening (Consorzio Tutela Grana Padano, 2011). For this reason samples below 9 months are indicated in quotation-marks.

Samples were lyophilised. Skimmed milk powder was used as a negative control. PageRuler™ Plus Prestained Protein Ladder (Fermentas, Vilnius, Lithuania) and lyophilised S-layer protein of *L. helveticus* MIMLh5 were used as positive controls. Then, samples were resuspended in the extraction buffer up to the concentration of 100 mg of lyophilisate per 1 mL of extraction buffer. The following extraction buffers were used: 5 M LiCl, 5 M urea, 1 % SDS, 4 % SDS, PBS buffer, 6 M GuHCl, 5 M LiCl with 8 M urea (ratio 1:1), 8 M urea. Extractions were performed at room temperature for 1 h in rotational agitation (Grant-bio PTR-30 rotator, Grant

Instruments, Cambridge, UK) except those of SDS, which were done at room temperature for 1 h as well as at 100 °C for 5 min. Subsequently, samples were centrifuged 5000 g at 40 °C for 30 min and freezed at –20 °C. If necessary, the solidified upper fat phase was removed with a wooden toothpick and the thawed intermediate phase (between precipitate of insoluble material (if present) and the upper fat) was taken for Western blottings.

2.3.17 Detection of S-layer protein in cheese samples by Western blotting

10–20 μL of cheese extracts (the maximum volume being absorbed by the membrane) were loaded as dots on methanol-activated polyvinylidene fluoride (PVDF) membrane (Immuno-Blot™ PVDF Membrane, absorbance rate 0.2 μm, Bio-Rad Laboratories, Richmond, CA, USA) and run at a constant voltage of 4 V (approximately 100 mA) at room temperature for 1 h on a Trans-Blot® SD Semi-Dry Transfer Cell Module (Bio-Rad Laboratories, Richmond, CA, USA) in 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) electroblotting buffer system (10 mM CAPS, pH 11, 10 % methanol). Membranes were blocked for at least 1 h in 5 % (w/v) non-fat dry milk in TBS (TRIS-buffered saline, pH 7.5) at room temperature. Blotting was done using anti-S-layer binder (anti-S-layer scFv-PhoA-6×His fusion) extracts in 1:200 dilution (in TBS with 0.05 % Tween-20) as primary antibody (incubation overnight at 8 °C in rotational agitation) and HRP-conjugated anti-His mouse monoclonal IgG1 (5Prime, VWR International, Helsinki, Finland) in 1:2000 dilution (in TBS with 0.05 % Tween-20) as secondary antibody (incubation for 1 h at room temperature in rotational agitation). The membrane was visualised (chromogenic detection) with 4-chloro-1-naphthol/3,3'-Diaminobenzidine (CN/DAB) Substrate Kit (Thermo Scientific, Rockford, USA) with two consecutive substrate exposures.

2.3.18 Statistical analysis

The significance of the results in the experiments for the evaluation of immunomodulatory activity of *L. helveticus* MIMLh5 S-layer protein and screening for the binding specificity of phage antibodies was analysed by unpaired heteroscedastic Student's *t* test with two-tailed distribution. Differences of $P < 0.05$ were considered to be significant.

2.4 RESULTS AND DISCUSSION

2.4.1 Extraction and purification of *L. helveticus* MIMLh5 S-layer protein

A generally employed method for the removal of S-layer protein from cell surfaces, LiCl extraction, was applied for the isolation of the S-layer protein from *L. helveticus* MIMLh5. It allowed obtaining S-layer protein extracts from different bacterial growth phases: early exponential (1 point, OD_{600}=0.5), mid-exponential (2 points, OD_{600}=1.5 and 2.5), and stationary phase (2 points, OD_{600}=4.0 and 5.0) (Figure 2.4.1A, indicated by arrows). The purity of the extracts was determined from the electrophoregrams of the SDS–PAGE analysis. The obtained gel demonstrates a single well defined band of approximately 45000 Da, corresponding to the S-layer protein of the analysed strain (Figure 2.4.1B; electrophoregram of the S-layer protein from mid-exponential growth phase (point of OD_{600}=2.5) is presented as an example). The purity of the extracted S-layer protein was confirmed by RP–HPLC analysis (Figure 2.4.1C).

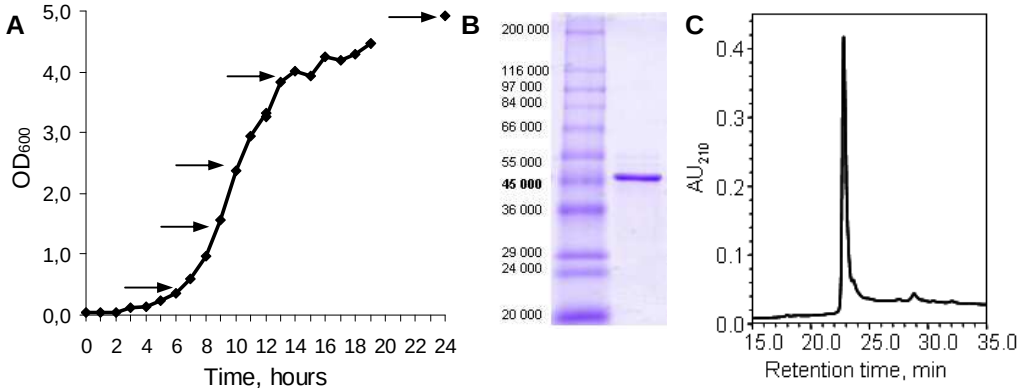

Figure 2.4.1. *Growth curve of* L. helveticus *MIMLh5 (A): arrows indicate growth phases the S-layer protein was extracted from. SDS–PAGE (B) and the RP–HPLC profile (C) of the S-layer protein from bacterial mid-exponential growth phase (OD_{600}=2.5).*

It was demonstrated that the purity of the extracted protein does not depend on the bacterial growth phase: SDS–PAGE and RP–HPLC analyses demonstrated one main band and the one main HPLC peak, respectively.

2.4.2 Evaluation of *in vitro* immunomodulatory activity of *L. helveticus* MIMLh5 S-layer protein

L. helveticus MIMLh5 S-layer protein was tested *in vitro* for a potential immunomodulatory activity. In this regard, it has been evaluated its capability to modulate the activation of transcription factor NF-κB in Caco-2 cells: in basal conditions as well as in the presence of pro-inflammatory stimulus of interleukin (IL)-1β. In basal conditions, the obtained results demonstrated a significant decrease of the bioluminescence, i.e. the activation of NF-κB, with S-layer samples from all bacterial growth phases (Figure 2.4.2A).

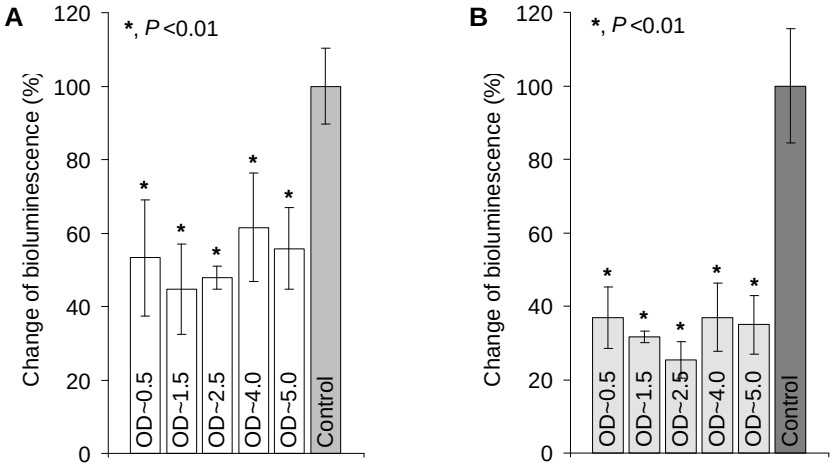

Figure 2.4.2. *Effects of S-layer protein extracts from different* L. helveticus *MIMLh5 growth phases (indicated by OD_{600}) on recombinant Caco-2 cells in basal conditions (A) and in the presence of co-stimulation with IL-1β (B). Luciferase activity is expressed as percentual change in relative luminescence units, assuming the control as 100 %. Control, Caco-2 incubated without S-layer protein. The values are the means (± standard deviation) of at least three independent experiments conducted in duplicate. An asterisk (*) indicates statistically significant differences compared to control.*

Further, the anti-inflammatory activity of all S-layer extracts was tested. For this reason, recombinant Caco-2 cells were co-stimulated with S-layer protein extracts from different bacterial growth phases and IL-1β, a prototypical pro-inflammatory cytokine that plays a central role in the amplification of inflammatory cascade. The addition of 2 ng mL^{-1} of IL-1β to Caco-2 culture medium caused nearly a twofold increase of NF-κB activity after 4 h incubation. After stimulation by IL-1β, all five S-layer extracts significantly reduced the NF-κB activation, while the control (EMEM medium with IL-1β, but without S-layer) did not have any significant effect (Figure 2.4.2B).

2.4.3 Determination of the *L. helveticus* MIMLh5 S-layer protein molecular mass

Determination of *L. helveticus* MIMLh5 S-layer protein size was performed by SDS–PAGE. It was calculated to be approximately 45 kDa (Figure 2.4.1B). To specify the molecular mass of the protein, an electrospray ionisation-mass spectrometry analysis after fractionation in ultra performance liquid chromatography (UPLC–ESI-MS) was performed. According to ESI-MS analysis the molecular mass of *L. helveticus* MIMLh5 S-layer protein was determined to be 43853 Da (Figure 2.4.3) in agreement with the size of the other known S-layer proteins from the genus *Lactobacillus* (Åvall-Jääskeläinen & Palva, 2005).

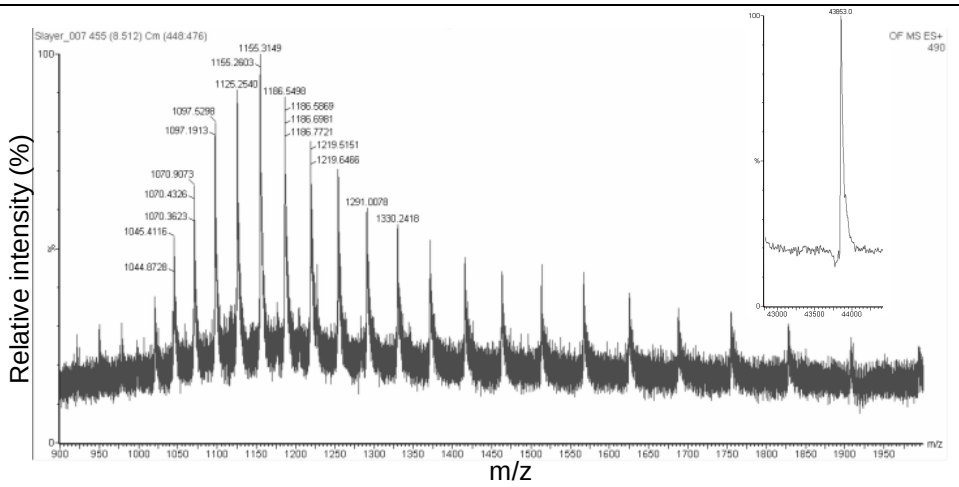

Figure 2.4.3. *ESI-MS spectrum of the* L. helveticus *MIMLh5 S-layer protein and reconstructed mass spectrum indicating an average mass value of 43853 Da.*

2.4.4 Prediction of the *L. helveticus* MIMLh5 S-layer protein sequence

PCR amplification with specific primers was used to amplify the S-layer protein gene from the genome of *L. helveticus* MIMLh5. A single PCR product was obtained from *L. helveticus* MIMLh5 chromosomal DNA (data not shown). The theoretical molecular mass obtained from the deduced amino acid sequence (Table 2.4.1) of the *L. helveticus* MIMLh5 S-layer protein was 46798 Da (with signal peptide) and 43853 Da (without signal peptide). The later corresponded to the molecular mass obtained by mass spectrometry analysis.

Table 2.4.1. *Amino acid composition of the S-layer protein of* L. helveticus *MIMLh5.*

Amino acid	Quantity	Molar ratio (%)
Ala (A)[H]	45	11.0
Arg (R)	5	1.2
Asn (N)	38	9.3
Asp (D)	25	6.1
Cys (C)	0	0.0
Gln (Q)	10	2.4
Glu (E)	9	2.2
Gly (G)	24	5.9
His (H)	3	0.7
Ile (I)[H]	22	5.4
Leu (L)[H]	12	2.9
Lys (K)	43	10.5
Met (M)[H]	3	0.7
Phe (F)[H]	11	2.7
Pro (P)[H]	13	3.2
Ser (S)	30	7.3
Thr (T)	51	12.4
Trp (W)[H]	0	0.0
Tyr (Y)	23	5.6
Val (V)[H]	43	10.5
Total	410	100

[H] hydrophobic amino acids (according Åvall-Jääskeläinen & Palva, 2005)

The S-layer protein of *L. helveticus* MIMLh5 has a high content of hydrophobic amino acid residues (36.3 %), which is a typical feature for all S-layer proteins. As observed for other S-layer proteins (Åvall-Jääskeläinen & Palva, 2005), also the MIMLh5 S-layer has a low content of sulphur-containing amino acids (0.7 %), which derives from methionine residues. A characteristic feature for all S-layer proteins, including the ones derived from *Lactobacillus*, is a high percentage of amino acids with hydroxyl groups (serine and threonine): 19.7 % in a case of MIMLh5 S-layer. From the basic amino acids, the lysine content of S-layer proteins is usually relatively high, 10 %, and this can also be noted for *L. helveticus* MIMLh5 S-layer protein (10.5 %).

The isoelectric point (pI) of the protein is high due to the presence of positively charged amino acid residues: it was calculated to be 9.39, which corresponds to the pI of the other known S-layer proteins from the genus *Lactobacillus* (pI 9.35 to 10.4, Åvall-Jääskeläinen & Palva, 2005). The protein is weakly soluble in aqueous solutions and is completely soluble in high ionic strength solutions such as 5 M LiCl and 8 M urea.

The overall sequence similarity of all up to date sequenced S-layer proteins within the genus *Lactobacillus* is very small. However, similarities between the S-layers of *L. helveticus* and *L. acidophilus* exist (Åvall-Jääskeläinen & Palva, 2005). A similarity search using the deduced amino acid sequence of the *L. helveticus* MIMLh5 S-layer protein was performed. It revealed rather high overall sequence similarity (84 % of consensus positions) with other S-layers of *L. helveticus* and three S-layer proteins of *L. acidophilus*. The dendrogram shows the presence of tree types of S-layer proteins within the species *L. helveticus* (Figure 2.4.4). The first type comprehends S-layer protein of *L. helveticus* MIMLh5 as well as the S-layer proteins of *L. acidophilus*. A high level of sequence homology was demonstrated at the N-terminal and C-terminal parts that are supposed to be responsible for membrane anchoring and self-assembly, respectively (Figure 2.4.5). These results are in agreement with previously published data about *L. helveticus* S-layer proteins (Gatti et al., 2005).

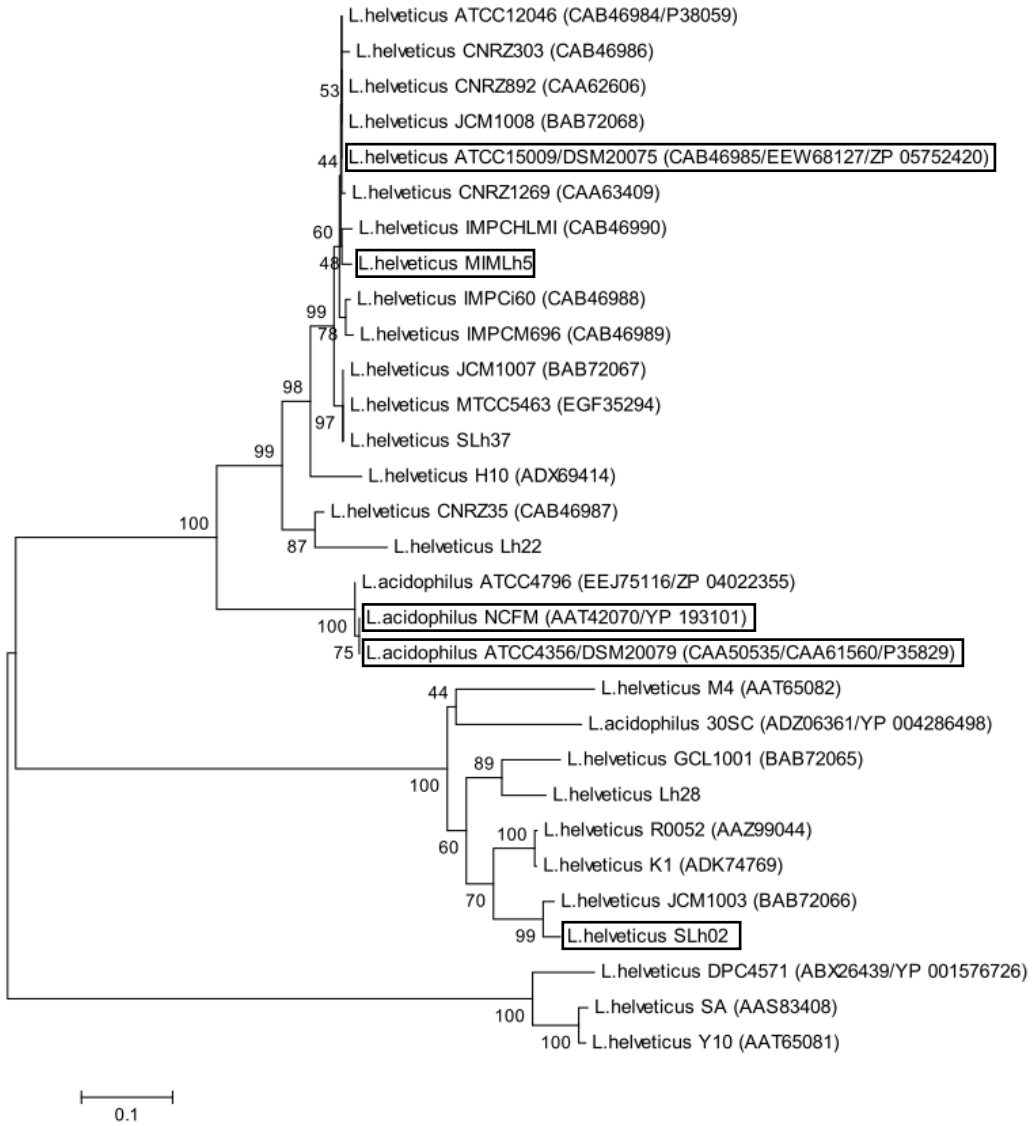

Figure 2.4.4. *Neighbour-joining dendrogram generated from the ClustalW alignment of the mature S-layer proteins most closely related to the S-layer protein of* L. helveticus *MIMLh5. Database accession numbers are indicated in parentheses. Bar indicates substitution/site. Bootstrap values of the main internodes (500 replicates) are shown. Proteins used in this study are circled.*

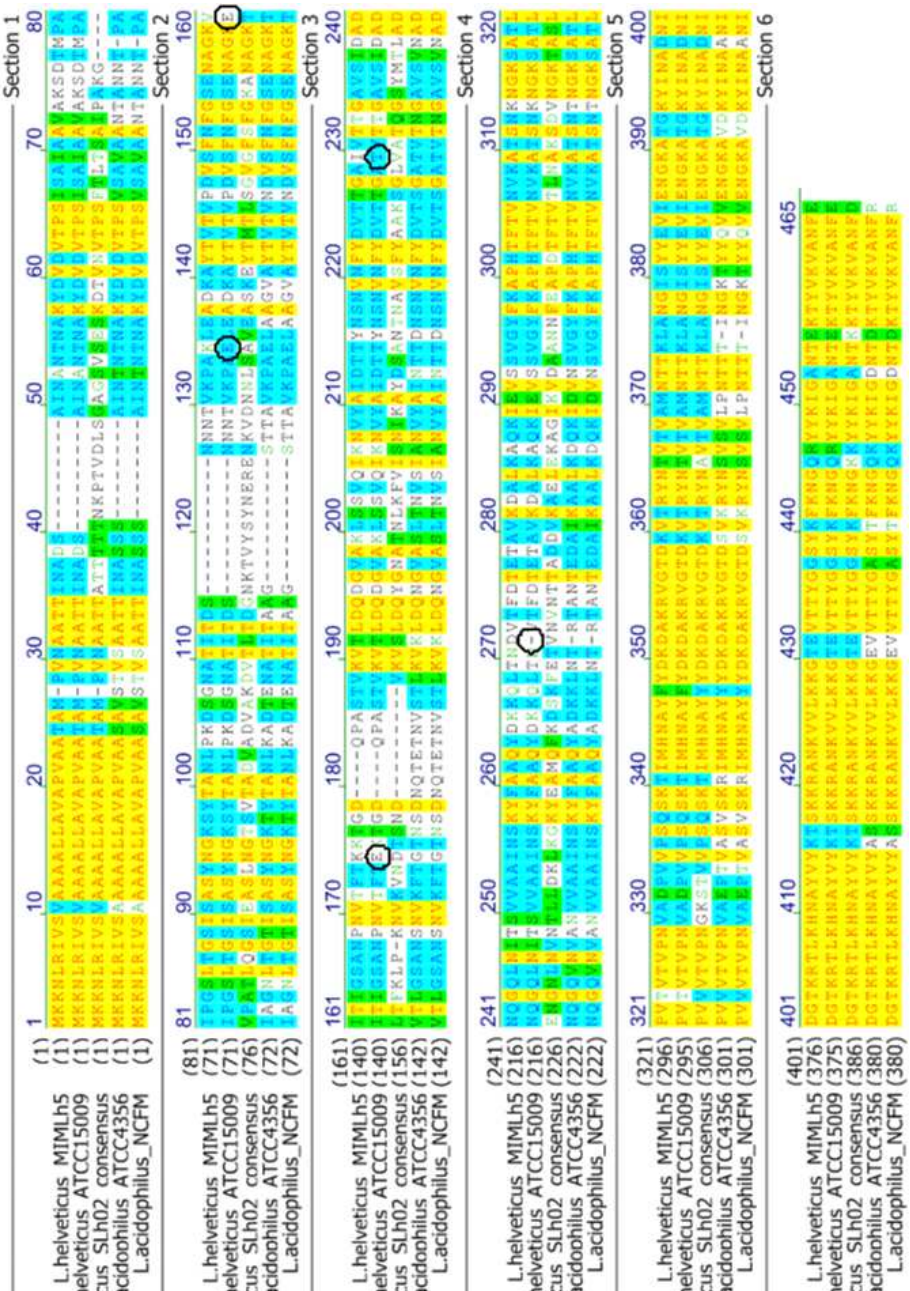

Figure 2.4.5. *Similarity of amino acid sequences of S-layer proteins from LAB used in this study. Differences of amino acids between the S-layer protein sequences of* L. helveticus *MIMLh5 and ATCC 15009 strains are indicated by circles.*

2.4.5 Selection and basic characterisation of scFv antibodies against *L. helveticus* MIMLh5 S-layer protein from phage displayed libraries

ScFv antibodies for *L. helveticus* MIMLh5 S-layer protein were obtained by phage display technology.

2.4.5.1 Immobilisation of *L. helveticus* MIMLh5 S-layer protein

The first step of scFvs selection against *L. helveticus* MIMLh5 S-layer protein was the purified protein immobilisation on the surface, i.e. coating of microtiter wells.

Two different surfaces, MaxiSorp™ and PolySorp™, were employed for coating. MaxiSorp™ and PolySorp™ surfaces were developed by Nunc (Roskilde, Denmark) for immunology assays. These surfaces are different in their physicochemical properties. The MaxiSorp™ surface is a modified, highly charged polystyrene surface with high affinity to molecules with polar or hydrophilic groups. The surface has a high binding capacity for proteins. The PolySorp™ surface is more hydrophobic than the MaxiSorp™ surface. It has high affinity to molecules of a more hydrophobic character. This surface is particularly suited to non-protein antigens. However, the S-layer protein of MIMLh5 contains 36.3 % of amino acids with hydrophobic side chains (Table 2.4.1). For this reason, also the PolySorp™ surface was used in our experiments.

For S-layer protein coating we selected passive coating strategy. This strategy was supposed to be advantageous because of the exceptional property of the S-layer protein to self-assembly on surfaces in its native conformation (Agave BioSystems, 2011; Horejs et al., 2011).

The insolubility of the protein in aqueous solutions introduced additional difficulties in coating procedure, as for coating the protein should be soluble. For this reason, we completely solubilised the protein in LiCl and then diluted with coating buffer.

For coating we selected a buffer system, which has a pI similar to that of the S-layer protein (9.39) and is compatible with LiCl-solubilised proteins: Tris–HCl buffer of pH 9.0

Subsequently, plates were blocked with two different blocking agents: milk (the traditional blocker used in phage selection) and bovine serum albumin (BSA, thinking to avoid the further possible cross-reactions of selected binders, which were supposed to be applied on milk-derived products).

There are no means to monitor the binding efficacy to the microtiter well of unknown target in the coating stage. Therefore, the comparison of binding efficacy to two different surfaces, MaxiSorp™ and PolySorp™, could be performed only in the subsequent stages, after the preselection of S-layer protein binders.

2.4.5.2 Selection, isolation and testing of binder pool

Selection and isolation. A mix of two synthetic phage display MolBind libraries pEB32x (scFv-p3 fusions), protein-directed and hapten-directed, was used for the selection of scFvs against the S-layer protein.

The pre-selection step on BSA-blocked plates without antigen (S-layer protein) was used to remove the phage binders, which showed specificity to the blocker as well as to the surface plastics.

Three rounds of panning were performed. The panning scheme is presented in Figure 2.4.6.

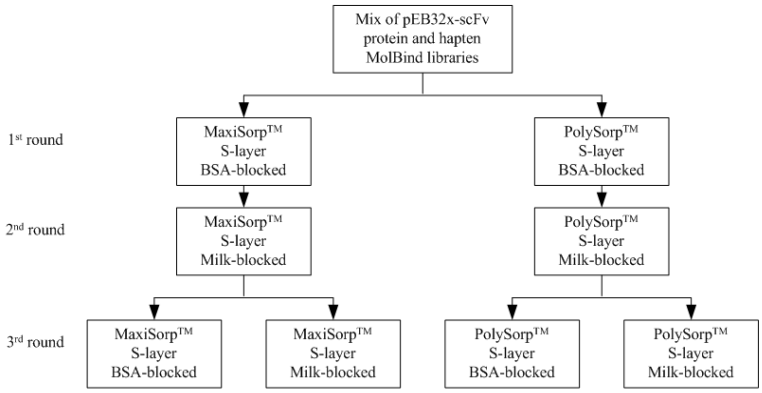

Figure 2.4.6. *Panning scheme for the selection of scFvs, specific for the* L. helveticus *MIMLh5 S-layer protein, from pEB32x-scFv MolBind libraries.*

The first panning was done on MaxiSorp™ and PolySorp™ plates, blocked with BSA. The second round was performed on MaxiSorp™ and PolySorp™ plates, blocked with milk, thinking to avoid the pre-selected binders at the first round, specific to milk proteins. The third round was done on MaxiSorp™ and PolySorp™ plates, blocked with milk or BSA, in order to have the maximal variety of obtained binders.

To monitor the enrichment of the panning process, input and output phages of each round were titrated (Table 2.4.2). The titre of the eluted phages dropped after the second round and increased strongly after the third.

Table 2.4.2. *Overview of antibodies' selection against the* L. helveticus *MIMLh5 S-layer protein.*

	Panning round							
	1		2		3			
	MaxiSorp BSA-blocked	PolySorp BSA-blocked	MaxiSorp Milk-blocked	PolySorp Milk-blocked	MaxiSorp BSA-blocked	MaxiSorp Milk-blocked	PolySorp BSA-blocked	PolySorp Milk-blocked
Phages applied (input)[a]	6.48×10^{12}	6.48×10^{11}	2.58×10^{11}	4.20×10^{11}	3.42×10^{11}	3.42×10^{11}	2.3×10^{11}	2.30×10^{11}
Phages eluted (output)	1.33×10^{8}	9.30×10^{7}	2.33×10^{6}	9.74×10^{6}	2.27×10^{7}	1.35×10^{8}	5.71×10^{6}	6.86×10^{7}
Background control[b]	–	–	3.82×10^{6}	1.08×10^{7}	6.81×10^{6}	1.51×10^{8}	8.54×10^{4}	3.85×10^{5}
Phage recovery percentage	0.21×10^{-4}	0.14×10^{-4}	0.90×10^{-5}	2.31×10^{-5}	0.66×10^{-4}	3.95×10^{-4}	2.48×10^{-5}	2.98×10^{-4}
Background[b]/Output	–	–	1.64	1.11	0.73	1.12	1.50×10^{-2}	5.51×10^{-3}

[a] The calculated empirical input.

[b] In phage selection experiments the background binding during the first panning round is not calculated, because the enrichment of specific binders after the first panning round is never visible.

The decreased background/output ratio and the increased phage recovery percentage (quantity of eluted phage divided by quantity of input phage) after the third panning round (in comparison with the second one) indicated that clones bound to the S-layer protein were enriched after 3 rounds of panning.

Testing. To screen for binding activity of selected four phage pools (scFv-p3 fusions) to the *L. helveticus* MIMLh5 S-layer protein the dissociation enhanced lanthanide fluoroimmunoasay (DELFIA®) was used and time resolved fluorescence (TRF) from Eu^{3+} was measured (Figure 2.4.7).

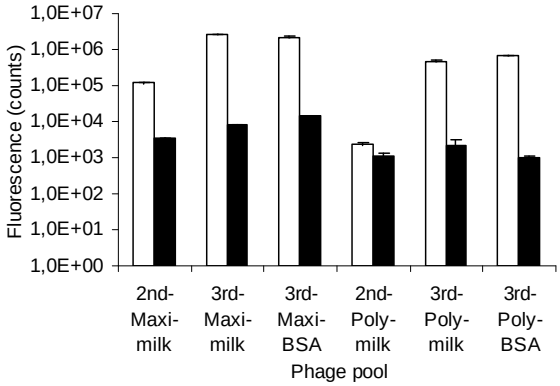

Figure 2.4.7. *Binding of the enriched phage pools (on MaxiSorp™ and PolySorp™ plates) to the immobilised S-layer protein after 2nd and 3rd rounds of panning. White columns represent time resolved fluorescence values (counts) of polyclonal anti-S-layer-scFv-p3 particles (diluted 1 : 10000) bound to immobilised S-layer protein of* L. helveticus *MIMLh5, blocked with BSA or milk; and black columns represent values of binding to blocking agent (BSA or milk). Time resolved fluorescence counts are presented on a logarithmic scale.*

S-layer protein-binding clones were strongly enriched after 3 rounds of panning on both surfaces, MaxiSorp™ and PolySorp™ (Figure 2.4.7). These results are in agreement with phage count data (Table 2.4.2).

After the 3rd panning round, both MaxiSorp™ pools and both PolySorp™ pools were similar among themselves for the S-layer protein binding. Therefore, individual clones were further isolated from one of the MaxiSorp™ and one of the PolySorp™ phage pools: 3rd-Maxi-BSA and 3rd-Poly-BSA.

2.4.5.3 Cloning and screening for individual binders

Subcloning. MaxiSorp™ BSA-blocked and PolySorp™ BSA-blocked scFv pools (scFv-p3 fusions) were subcloned into pLK06H for expression as periplasm-expressed His-tagged PhoA (anti-S-layer-scFv-PhoA-6×His) fusions (Figure 2.4.8).

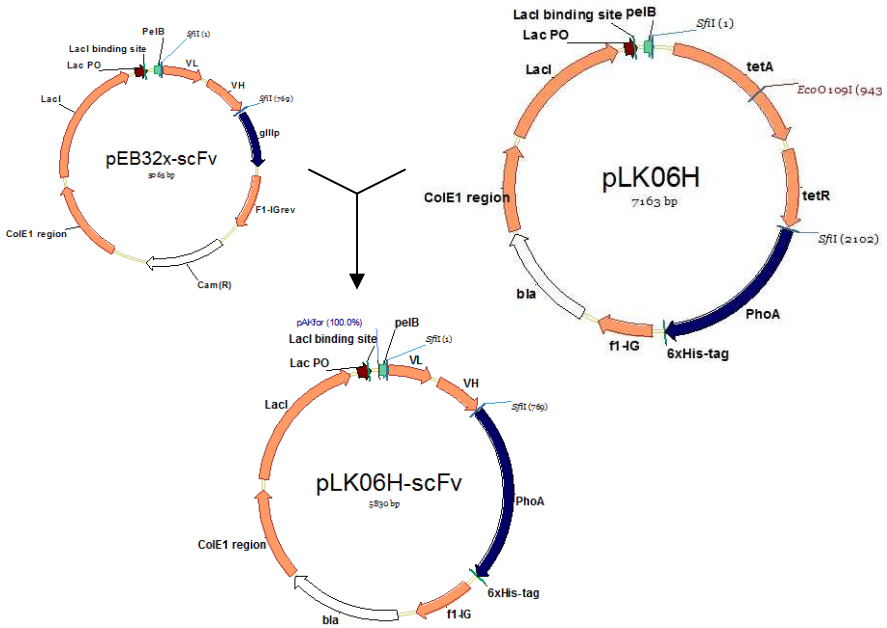

Figure 2.4.8. *Cloning scheme to obtain individual anti-S-layer-scFv binders: PhoA fusion.*

Single clones of transformants were cultivated in the wells of a microtiter plate and 84 individual scFv-PhoA-6×His binders were produced.

Screening for the binding activity of individual phage antibodies to purified S-layer protein. In order to evaluate the specificity of 84 scFv- PhoA binders for detecting the *L. helveticus* MIMLh5 purified S-layer protein, an AP chromogenic ELISA assay was performed on immobilised S-layer protein on MaxiSorp™ plates (Figure 2.4.9).

Thirty seven individual clones (44 %), in which scFv exhibited the strongest interaction with S-layer protein, were selected.

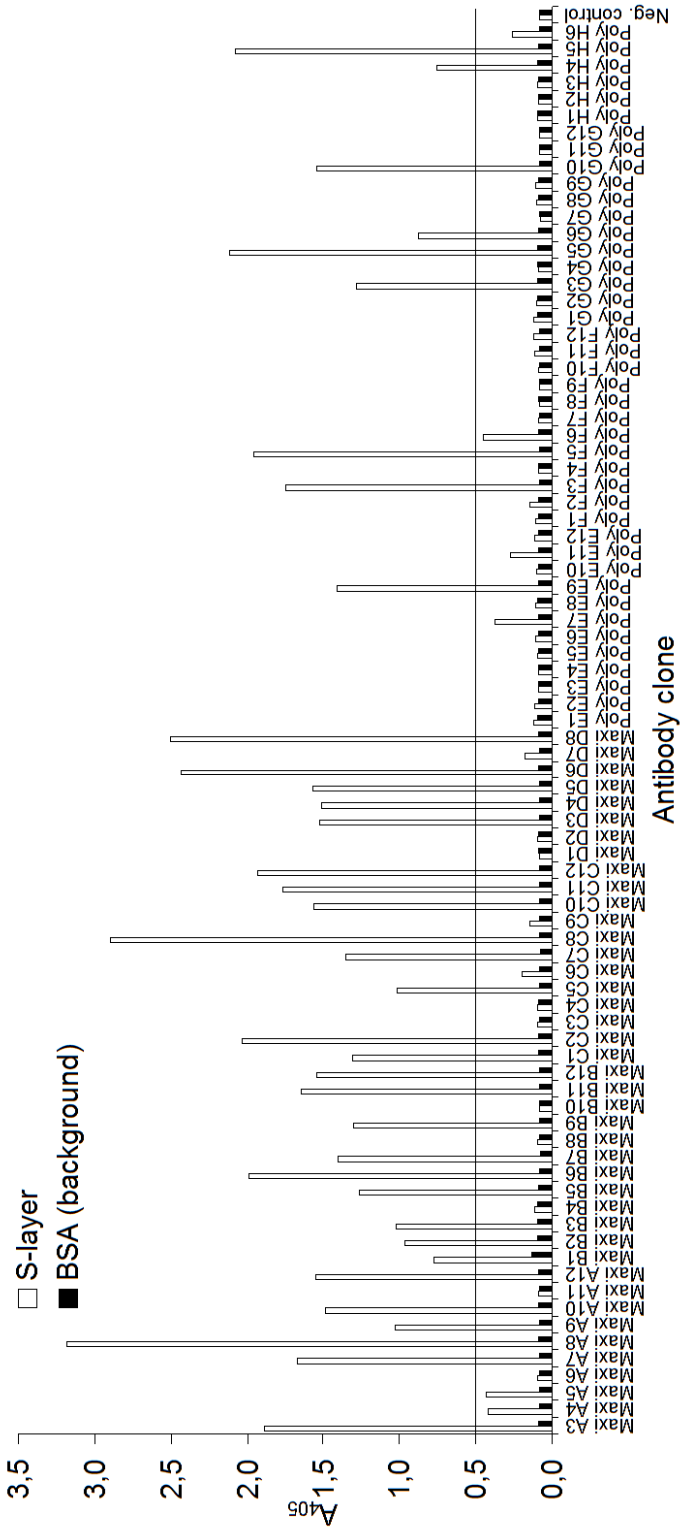

Figure 2.4.9. *Characteristics of individual anti-S-layer protein scFv binders (step 1). White columns represent pNPP chromogenic assay absorbance at 405 nm of monoclonal anti-S-layer-scFv-PhoA-6×His particles bound to immobilised S-layer protein of* L. helveticus *MIMLh5, blocked with BSA; black columns represent binding to blocking agent (BSA). Antibody selection threshold (as described in chapter 2.3.11) is indicated with a horizontal line.*

Screening for the binding activity of monoclonal scFv antibodies to S-layer protein-containing (Slp+) bacterial cells. A similar AP chromogenic ELISA assay was performed with 37 selected scFv-PhoA binders in order to evaluate their specificity towards non-purified S-layer protein present on intact bacterial cells of *L. helveticus* MIMLh5. To this aim, we developed an assay in which antibodies were added to a suspension of bacterial cells (*in suspension* assay) (Figure 2.4.10).

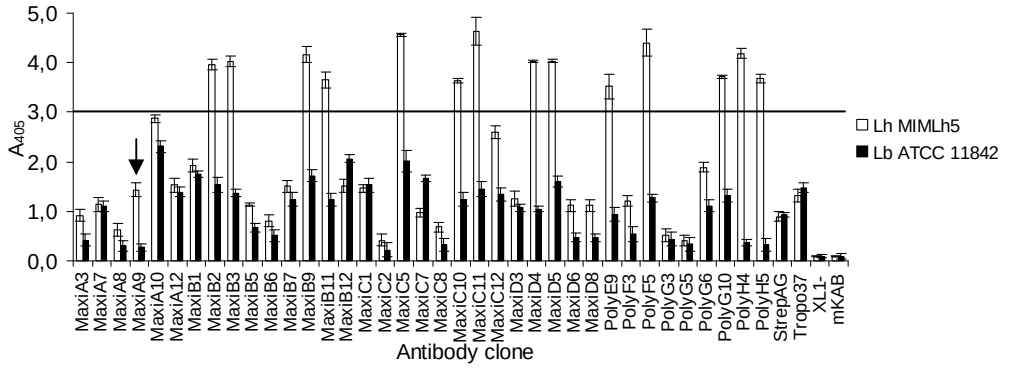

Figure 2.4.10. *Characteristics of individual anti-S-layer protein scFv binders (step 2). White columns represent pNPP chromogenic in suspension assay absorbance at 405 nm of monoclonal anti-S-layer-scFv-PhoA-6×His particles bound to* L. helveticus *MIMLh5 cells; black columns represent binding to* L. delbrueckii *subsp.* bulgaricus *ATCC 11842 (Lb11842; Slp–) cells. Antibody selection threshold (as described in chapter 2.3.12) is indicated with a horizontal line and an arrow.*

Assay buffer (mKAB) and the lysate of *E. coli* XL1-Blue cells (which do not express any binder) were included as negative controls to demonstrate the pNPP background absorbance. StrepA G09-scFv and Tropo37-scFv binders that were obtained from pannings with proteins, not related to the S-layer protein, were used as negative controls to demonstrate the preliminary background binding signal of pNPP chromogenic assay.

It was found that fifteen out of 37 scFv binders (40.5 %) were specific binders to *L. helveticus* MIMLh5 (Slp+), but not to *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (Slp–, a species phylogenetically closely related to *L. helveticus*, but lacking the S-layer protein). Fourteen of them had high relative expression levels

Finally, *in suspension* assay was performed with diverse S-layer containing bacterial cells: *L. helveticus* MIMLh5 (contains our analysed S-layer protein), *L. helveticus* ATCC 15009 (has the S-layer protein similar to that of MIMLh5, and diverse from MIMLh5 in only five amino acids), *L. helveticus* SLh02 (harbours the S-layer protein, belonging to the second type of S-layer proteins, related to that of *L. helveticus* MIMLh5; Figure 2.4.4), *L. acidophilus* ATCC 4356 and NCFM (two identical S-layer proteins, phylogenetically related to that of *L. helveticus* MIMLh5) (Figure 2.4.5). *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 is a bacterial species phylogenetically related to *L. helveticus*, but it does not have the S-layer protein (Slp–). Fifteen Slp+ bacterial cell-specific scFv binders were analysed (Figure 2.4.11).

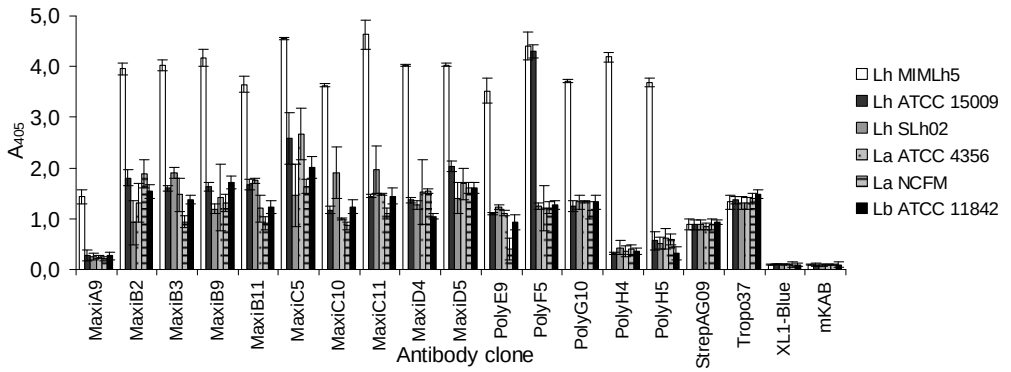

Figure 2.4.11. *Characteristics of individual anti-S-layer protein scFv binders (step 3). White columns represent pNPP chromogenic assay absorbance at 405 nm of monoclonal anti-S-layer-scFv-PhoA-6×His particles bound to* L. helveticus *MIMLh5 cells; black columns represent binding to* L. delbrueckii *subsp.* bulgaricus *ATCC 11842 (Slp–) cells; diverse grey columns represent absorbance at 405 nm of monoclonal anti-S-layer-scFv-PhoA-6×His particles bound to bacterial cells harbouring phylogenetically-related S-layer proteins (Figure 2.4.5).*

The assay demonstrated that fourteen scFv antibodies recognised only *L. helveticus* MIMLh5 cells. One antibody, anti-S-layer scFv-PhoA-6×His PolyF5, was less specific and recognised both, *L. helveticus* MIMLh5 and *L. helveticus* ATCC 15009 S-layer proteins present on bacterial cells. It is worth noting that binders PolyH4 and PolyH5 had very low background binding level.

Sequencing. DNA of the fifteen monoclonal binders, in which anti-S-layer-scFv-PhoA exhibited the best interaction with *L. helveticus* MIMLh5 S-layer protein, was sequenced: MaxiA9, B2, B3, B9, B11, C5, C10, C11, D4, D5, PolyE9, G10, H4, H5, F5. Six of them were different: PolyH4, PolyH5, PolyF5, MaxiC5, MaxiC11 and a group of ten identical binders (MaxiA9, B11, B2, B3, B9, C10, D4, D5, PolyE9, PolyG10) represented by MaxiB9, which according specificity to bacterial cells, harbouring phylogenetically-related S-layer proteins, seems to show the highest affinity and the highest expression level (Figure 2.4.11). MaxiC5 was discarded, because the phagemid formed a concatemer (data not shown). The deduced amino acid sequences were aligned with human synthetic scFv (framework scFv gene) (Figure 2.4.11), on the basis of which the pEB32x MolBind libraries were constructed (Brockmann et al., 2011).

The framework gene and anti-S-layer-scFvs differed in the CDR1 and CDR3 regions of both heavy and light chains and CDR2 of heavy chain, which corresponds with the phage library design rules (Brockmann et al., 2011). The only PolyF5 binder was selected from pEB32x MolBind protein-directed library, because it has a tryptophan (W) as the last amino acid in the CDR-L3 loop, the fingerprint of this library. Accordingly, the remaining binders were selected from pEB32x MolBind hapten-directed library.

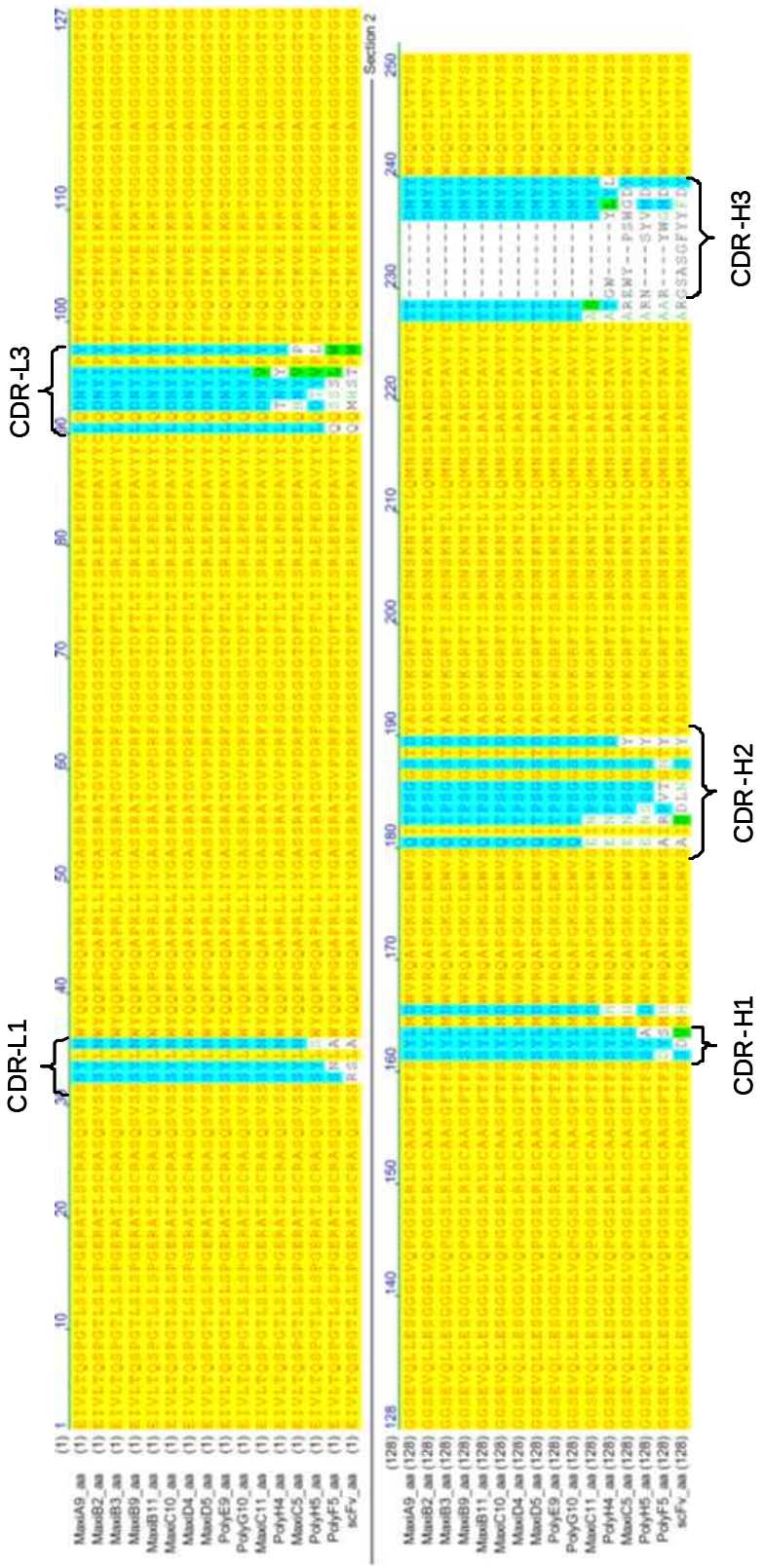

Figure 2.4.12. *Alignment of scFv sequences of fifteen selected S-layer protein-specific binders with a framework scFv protein. Randomised complementarity-determining regions (CDRs) of scFvs' light (L) and heavy (H) chains are indicated.*

2.4.5.4 Determination of anti-S-layer-scFv antibodies' binding specificity

A more detailed analysis of the anti-S-layer scFvs binding pattern was achieved by determining anti-S-layer-scFv binders' specificity by Western blotting. For this, five selected scFvs (MaxiB9, MaxiC11, PolyF5, PolyH5 and PolyH5) were expressed in liquid cultures as 6×His-tagged PhoA fusions. For **denaturing conditions**, they were applied as primary antibodies on LAB lysates (selected as described above), separated by SDS–PAGE (denaturing conditions) and blotted on a PVDF membrane. The binding of scFvs was detected by an anti-His HRP-conjugate and visualised with a CN/DAB Liquid Substrate System by chromogenic detection (Figure 2.4.13).

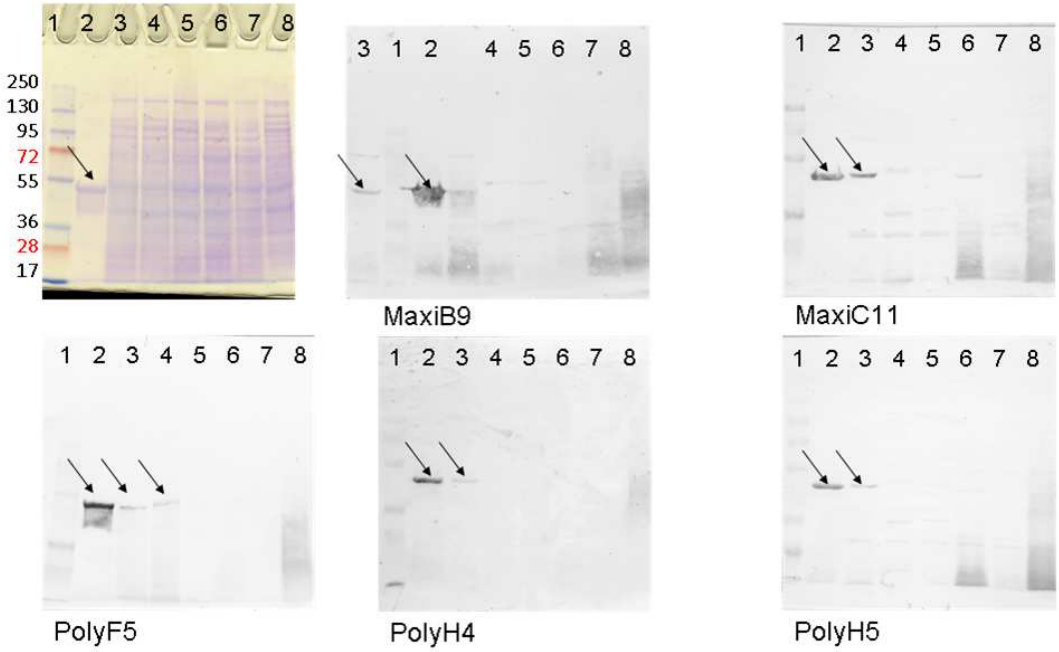

Figure 2.4.13. *Specificity of anti-S-layer scFvs towards purified S-layer protein of* L. helveticus *MIMLh5 and S-layer protein-containing LAB lysates in denaturing conditions as revealed by Western blot. The SDS–PAGE gel was stained with Coomassie brilliant blue G-250 to reveal the whole protein profile. CN/DAB substrate was used to visualise the binding of scFvs (MaxiB9, MaxiC11, PolyF5, PolyH4, PolyH5). Lane 1, protein molecular weight ladder; lane 2,* L. helveticus *MIMLh5 purified S-layer protein; lanes 3–7, lysates of Slp+ LAB strains:* L. helveticus *MIMLh5 (lane 3);* L. helveticus *ATCC 15009 (lane 4);* L. helveticus *SLh02 (lane 5);* L. acidophilus *ATCC 4356 (lane 6);* L. acidophilus *NCFM (lane 7); lane 8, lysate of Slp–* L. delbrueckii *subsp.* bulgaricus *ATCC 11842 strain, used as a negative control. Sharp bands revealed by scFvs are indicated by arrows.*

The purified S-layer protein from *L. helveticus* MIMLh5 (lane 2) was separated in the SDS–PAGE as a single band (Figure 2.4.13). LAB cell lysates were separated into a mixture of bands (lanes 3–8), representing the total bacterial protein content.

Binders MaxiB9, MaxiC11, PolyH4, PolyH5 strongly bound to the purified S-layer protein of the *L. helveticus* MIMLh5 (Figure 2.4.13, lane 2) as well as to the approximately 45 kDa band, representing the S-layer protein in the lysate of the MIMLh5 strain (Figure 2.4.13, lane 3). ScFv PolyF5 bound not only to the purified S-layer protein of *L. helveticus* MIMLh5 and MIMLh5 cell lysate, but also to the lysate of *L. helveticus* ATCC 15009 (Figure 2.4.13, PolyF5, lane 4). This result is in agreement with the data obtained previously with *in suspension* assay (Figure 2.4.11).

All binders (especially MaxiB9, MaxiC11 and PolyH5) showed relatively high background binding. It is not surprising, since antibodies, selected in pannings by phage display technology, normally do not demonstrate very high binding affinity. To increase the binder's affinity, the subsequent step of affinity maturation is required (Hoogenboom & Chames, 2000; Barderas et al., 2008). However, this was not a task of the present study.

Summarising, the binding specificity of five different anti-S-layer binders was tested by Western blotting in denaturing conditions. Four of them, MaxiB9, MaxiC11, PolyH4 and PolyH5, were specific to only the denatured S-layer of *L. helveticus* MIMLh5 strain. PolyF5 was less specific and recognised the denatured S-layer protein of both, *L. helveticus* MIMLh5 and ATCC 15009 strains.

To test the binding specificity of five different anti-S-layer binders to the S-layer in **native conditions**, anti-S-layer scFv-6×His-tagged PhoA fusions were applied as primary antibodies on non-denatured LAB lysates, dot-blotted on a PVDF membrane and visualised as described above (Figure 2.4.14).

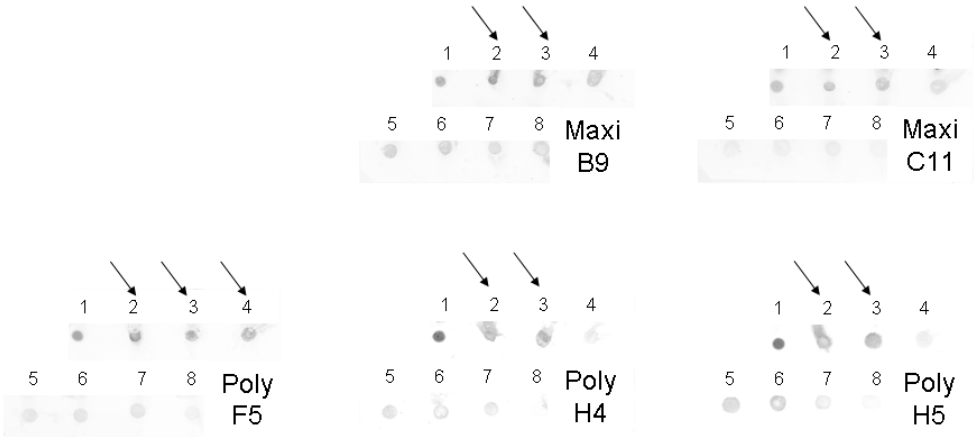

Figure 2.4.14. *Specificity of anti-S-layer scFvs towards purified S-layer protein of* L. helveticus *MIMLh5 and S-layer protein-containing LAB lysates in native conditions as revealed by Western dot-blot. CN/DAB substrate was used to visualise the binding of scFvs (MaxiB9, MaxiC11, PolyF5, PolyH4, PolyH5). 1, protein molecular weight ladder; 2,* L. helveticus *MIMLh5 purified S-layer protein; 3–7, lysates of Slp+ LAB strains:* L. helveticus *MIMLh5 (dot 3);* L. helveticus *ATCC 15009 (dot 4);* L. helveticus *SLh02 (dot 5);* L. acidophilus *ATCC 4356 (dot 6);* L. acidophilus *NCFM (dot 7); 8, lysate of Slp–* L. delbrueckii *subsp.* bulgaricus *ATCC 11842 strain, used as a negative control. Sharp dots revealed by scFvs are indicated by arrows.*

MaxiC11, PolyH4 and PolyH5 strongly bound to the *L. helveticus* MIMLh5 purified S-layer protein and to the lysates of *L. helveticus* MIMLh5 and ATCC 15009. MaxiB9 demonstrated high binding to the lysates of all tested LAB strains. This could be justified by the binder's low specificity in native conditions. PolyF5, as in the case of denaturing conditions, was specific to both, *L. helveticus* MIMLh5 and ATCC 15009, S-layer proteins.

As in the case of denaturing conditions, also in native conditions all binders showed relatively high background binding. It is not surprising, taking into account not only the binders' low affinity, but also the presence of other histidine-containing bacterial proteins that exist in the intact bacterial protein extract, which is concentrated in one dot (but not fractionated as in the case of denaturing conditions). It is worth noting that in the case of PolyH4 binding to the lysate of *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 strain (Slp–), the dot was almost invisible. These results confirm the high specificity of scFv PolyH4 to the S-layer protein of *L. helveticus* MIMLh5. Therefore, this binder was further tested for sensitivity (chapter 2.4.5.5) and used to detect the *L. helveticus* MIMLh5 S-layer protein on Grana Padano cheese samples.

2.4.5.5 Determination of anti-S-layer-scFv antibodies' sensitivity

In order to test the anti-S-layer scFv PolyH4 sensitivity, the *L. helveticus* MIMLh5 S-layer protein detection limit with this antibody was determined. For this reason, different quantities of the *L. helveticus* MIMLh5 S-layer protein solution were spotted on the membrane: 1 μg, 500 ng, 100 ng, 10 ng, 1 ng, 500 pg, 100 pg, 10 pg, and 1 pg. The anti-S-layer scFv PolyH4 was used for Western blotting as primary antibody, and the chromogenic detection was performed as described in chapter 2.3.15. The blotogram revealed the presence of the *L. helveticus* MIMLh5 S-layer protein in the range of quantities starting from 500 pg – 1 ng (Figure 2.4.15).

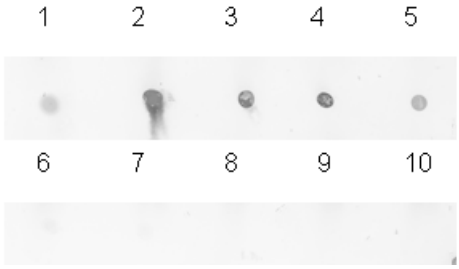

Figure 2.4.15. *Sensitivity of anti-S-layer scFv PolyH4 against different quantities of the* L. helveticus *MIMLh5 S-layer protein. 1, protein molecular weight ladder; 2, 1 μg; 3, 500 ng; 4, 100 ng; 5, 10 ng; 6, 1 ng; 7, 500 pg; 8, 100 pg; 9, 10 pg; 10, 1 pg of the* L. helveticus *MIMLh5 S-layer protein.*

This result is in agreement with indirect CN/DAB Liquid Substrate System chromogenic detection limit.

2.4.6 Detection of *L. helveticus* S-layer protein in Grana Padano samples

L. helveticus MIMLh5 was isolated from NWS used in the production of Grana Padano, a protected-designation-of-origin cheese, produced in specific areas of Northern Italy (Italian Presidential Decree, 1955). The monitoring of bacterial population dynamics during Grana Padano cheese productions was documented (Zago et al., 2007), and the persistence of *L. helveticus* strains in its NWS and the first maturation phases was described (Gatti et al., 2004; Lazzi et al., 2004; Rossetti et al., 2008; Santarelli et al., 2008). However, no information is present concerning the fate of the immunologically important S-layer protein of this bacterium during the Grana Padano cheese production and ripening process.

To select the optimal conditions for the S-layer protein extraction from cheese samples, we used the NWS, as a sample with the highest probability to possess the largest amounts of *L. helveticus* S-layer protein (Zago et al., 2007). We performed S-layer extraction from NWS with

several different reagents: (1) 5 M LiCl (Lortal et al., 1992; Smit et al., 2001), (2) 1 % and 4 % SDS at room temperature and boiling (extraction protocol used to obtain total bacterial protein extracts; Austin et al., 1990), (3) 5 M and 8 M urea (Podleśny et al., 2011), (4) 6 M guanidine hydrochloride (GuHCl, Boot et al., 1993; Sillanpää et al., 2000; Garrote et al., 2004), (5) combining 5 M LiCl and 8 M urea, (6) phosphate-buffered saline (PBS). The obtained extracts were spotted on the blotting membrane, and the S-layer protein was detected using a single anti-S-layer scFv PolyH4 as the most specific binder with the lowest background binding as revealed previously (Figure 2.4.13 and Figure 2.4.14). Specificity of anti-S-layer scFv PolyH4 against Grana Padano NWS extracts, obtained after treatment in different conditions, is presented in Figure 2.4.16.

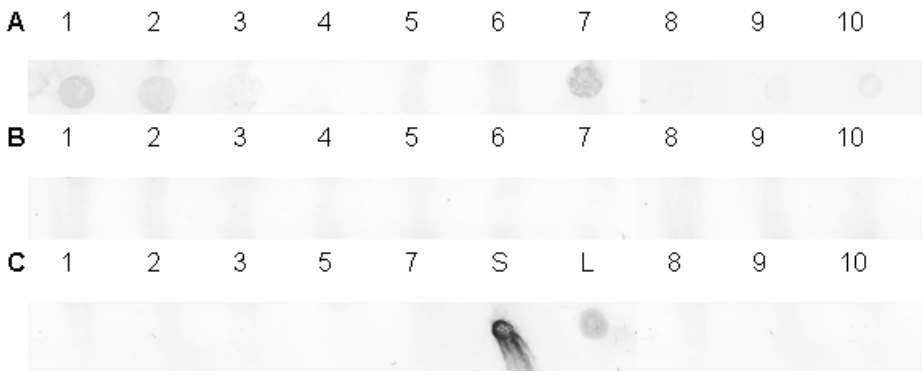

Figure 2.4.16. *Selection of S-layer protein extraction conditions. Specificity of anti-S-layer scFv PolyH4 against Grana Padano NWS extracts (A), obtained after treatment in different conditions: 1, 5 M LiCl room temperature (R/T), 2, 5 M urea R/T, 3, 1 % SDS R/T, 4, 1 % SDS 100 °C, 5, 4 % SDS R/T, 6, 4 % SDS 100 °C, 7, PBS R/T, 8, 6 M GuHCl R/T, 9, 5 M LiCl and 8 M urea R/T, 10, 8 M urea R/T. Purified* L. helveticus *MIMLh5 S-layer protein (S) was used as positive control. Protein molecular weight ladder (L) was used as Western blotting positive control. Milk (B) and extraction buffers (C) were used as negative controls.*

6 M GuHCl demonstrated too harsh conditions by visibly destroying the blotting membrane. SDS extraction gave negative results (Figure 2.4.16A 3–6). After extraction with 5 M LiCl, 5 M urea, 5 M LiCl with 8 M urea, and 8 M urea it was possible to visualise the signals (Figure 2.4.16A 1–2, 9–10). PBS extract gave a signal, but formed a particulate material on the membrane (Figure 2.4.16A 7). No detectable signal from the negative controls proved that all the observed positive dots must be binding from scFvs, and not unspecific phage particle binding (Figure 2.4.16B 1–10). However, the strongest positive signal was obtained with the S-layer-specific extraction agent, 5 M LiCl (Figure 2.4.16A 1). For this reason, the extraction buffer 5 M LiCl was selected to extract the S-layer protein from all selected Grana Padano samples.

NWS, curd and seven “cheese” samples from five Grana Padano cheese maturation phases were selected as indicated in chapter 2.3.16. S-layer protein was extracted with 5 M LiCl and the extracts were applied on the blotting membrane. Anti-S-layer scFv PolyH4 was used as primary antibody for the S-layer protein detection (Figure 2.4.17).

Figure 2.4.17. *Detection of* L. helveticus *MIMLh5 S-layer protein in Grana Padano NWS, curd and "cheese" samples with anti-S-layer scFv PolyH4 binder: 1, NWS, 2, curd, 3 GP-2.0, 4, GP-3.5, 5, GP-3.6, 6, GP-4.0, 7-9, GP-5.0 months, 10, negative control (milk).*

Western blot analysis with anti-S-layer scFv PolyH4 binder revealed the presence of *L. helveticus* MIMLh5 S-layer protein in Grana Padano NWS, curd and "cheese" samples from maturation phases of 2.0, 3.5, 3.6, 4.0 and 5.0 months (Figure 2.4.17). The most intense dot was that of the NWS, as it should contain the highest amount of *L. helveticus* cells (Zago et al., 2007). Different dot intensities of 5-month samples could be explained by differences between manufacturer the samples were taken from.

These results showed promising applications of anti-S-layer scFv PolyH4 binder for the detection of immunologically important *L. helveticus* S-layer protein at nanogram quantities in Grana Padano cheese and other food matrices.

2.5 CONCLUSIONS

According to the tasks described in the chapter 2.2, the following conclusions were drawn:

1. Efficient extraction protocol was set up to obtain pure extracts of *L. helveticus* MIMLh5 S-layer protein.
2. *L. helveticus* MIMLh5 S-layer protein mass was found to be 43853 Da.
3. Phage display technology was successfully employed to select the *L. helveticus* MIMLh5 S-layer protein-specific binders.
4. Five different anti-S-layer scFv binders were selected from phage display libraries. Four of them, MaxiB9, MaxiC11, PolyH4 and PolyH5, were specific to only the S-layer of *L. helveticus* MIMLh5 strain. PolyF5 recognised the S-layer protein of *L. helveticus* MIMLh5 and ATCC 15009 strains.
5. On this basis, an assay for the detection of *L. helveticus* MIMLh5 S-layer protein in Grana Padano cheese was developed using anti-S-layer scFv PolyH4 binder.

The expressed scFvs were applied in Western blotting analysis and showed promising application in detection of immunologically important *L. helveticus* MIMLh5 S-layer protein at nanogram quantities in Grana Padano cheese and other food matrices.

A following study is now in progress to identify the presence and the immunomodulatory activity of the S-layer protein in Grana Padano cheese ripened more than 5 months and in Grana Padano cheese submitted to simulated gastrointestinal digestion.

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Ačiū Jonui: už viską, kas tikrai nesutelpa į šias eilutes ir į visą šį kūrinį...

Milda Stuknytė

Milan, December 2011

APPENDIX 1. COPIES OF ABSTRACTS OF PAPERS, ORAL COMMUNICATIONS AND POSTERS

1. Guglielmetti S, Taverniti V, Minuzzo M, Arioli S, **Stuknyte M**, Karp M, Mora D, 2010, Oral bacteria as potential probiotics for the pharyngeal mucosa. Appl Environ Microbiol 76:3948-3958.

2. Guglielmetti S, Taverniti V, Minuzzo M, Arioli S, Zanoni I, **Stuknyte M**, Granucci F, Karp M, Mora D, 2010, A dairy bacterium displays *in vitro* probiotic properties for the pharyngeal mucosa by antagonizing group A streptococci and modulating the immune response. Infect Immun 78: 4734-4743.

3. **Stuknyte M**, De Noni I, Guglielmetti S, Minuzzo M, Mora D, 2011, Potential immunomodulatory activity of bovine casein hydrolysates produced after digestion with proteinases of lactic acid bacteria. Int Dairy J 21:763-769.

4. **Stuknyte M**, Brockmann E-C, Huovinen T, Taverniti V, Guglielmetti S, Mora D, De Noni I, Lamminmäki U, manuscript, Selection of a Single-Chain Variable Fragment Antibody against *Lactobacillus helveticus* MIMLh5 S-layer Protein from Phage Displayed Libraries.

5. **Stuknyte M**, Development of bioprocesses for the production of food protein hydrolysates containing bioactive peptides. In Proceedings of the 14th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Oristano, Italy, 16-18 September, 2009, pp. 446-447.

6. **Stuknyte M**, Development of bioprocesses for the production of food protein hydrolysates containing bioactive peptides. In Proceedings of the 15th Workshop on the Development in the Italian PhD Research on Food Science Technology and Biotechnology, Portici, Italy, 15-17 September, 2010, pp. 299-300.

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8. **Stuknyte M**, Immunomodulatory activity of bovine casein hydrolysates produced after digestion with proteinases of lactic acid bacteria. In Proceedings of the 16th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Lodi, Italy, 21-23 September, 2011, pp. 181-185.

9. **Stuknyte M**, Brockmann E-C, Huovinen T, Taverniti V, Guglielmetti S, Mora D, De Noni I, Lamminmäki U, *Lactobacillus helveticus* S-layer protein specific binders from antibody libraries. In Book of Abstracts of The 4th Interdepartmental Twinning Symposium, Tammela, Finland, 28 September – 2 October, 2011, p. 17.

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Oral Bacteria as Potential Probiotics for the Pharyngeal Mucosa▽

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The research described here was aimed at the selection of oral bacteria that displayed properties compatible with their potential use as probiotics for the pharyngeal mucosa. We included in the study 56 bacteria newly isolated from the pharynges of healthy donors, which were identified at the intraspecies level and characterized *in vitro* for their probiotic potential. The experiments led us to select two potential probiotic bacterial strains (*Streptococcus salivarius* RS1 and ST3) and to compare them with the prototype oral probiotic *S. salivarius* strain K12. All three strains efficiently bound to FaDu human epithelial pharyngeal cells and thereby antagonized *Streptococcus pyogenes* adhesion and growth. All were sensitive to a variety of antibiotics routinely used for the control of upper respiratory tract infections. Immunological *in vitro* testing on a FaDu layer revealed different responses to RS1, ST3, and K12. RS1 and ST3 modulated NF-κB activation and biased proinflammatory cytokines at baseline and after interleukin-1β (IL-1β) induction. In conclusion, we suggest that the selected commensal streptococci represent potential pharyngeal probiotic candidates. They could display a good degree of adaptation to the host and possess potential immunomodulatory and anti-inflammatory properties.

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A Dairy Bacterium Displays *In Vitro* Probiotic Properties for the Pharyngeal Mucosa by Antagonizing Group A Streptococci and Modulating the Immune Response▽

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The probiotic approach represents an alternative strategy in the prevention and treatment of infectious diseases, not only at the intestinal level but also at other sites of the body where the microbiota plays a role in the maintenance of physiological homeostasis. In this context, we evaluated *in vitro* the potential abilities of probiotic and dairy bacteria in controlling *Streptococcus pyogenes* infections at the pharyngeal level. Initially, we analyzed bacterial adhesion to FaDu hypopharyngeal carcinoma cells and the ability to antagonize *S. pyogenes* on FaDu cell layers and HaCat keratinocytes. Due to its promising adhesive and antagonistic features, we studied the dairy strain *Lactobacillus helveticus* MIMLh5, also through *in vitro* immunological experiments. First, we performed quantification of several cytokines and measurement of NF-κB activation in FaDu cells. MIMLh5 efficiently reduced the induction of interleukin-6 (IL-6), IL-8, and tumor necrosis factor alpha (TNF-α), in a dose-dependent manner. After stimulation of cells with IL-1β, active NF-κB was still markedly lowered. Nevertheless, we observed an increased secretion of IL-6, gamma interferon (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF) under these conditions. These effects were associated with the ability of MIMLh5 to enhance the expression of the heat shock protein coding gene *hsp70*. In addition, MIMLh5 increased the GM-CSF/G-CSF ratio. This is compatible with a switch of the immune response toward a TH1 pathway, as supported by our observation that MIMLh5, once in contact with bone marrow-derived dendritic cells, triggered the secretion of TNF-α and IL-2. In conclusion, we propose MIMLh5 as a potential probiotic bacterium for the human pharynx, with promising antagonistic and immunomodulatory properties.

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Potential immunomodulatory activity of bovine casein hydrolysates produced after digestion with proteinases of lactic acid bacteria

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ABSTRACT

During hydrolysis of bovine milk caseins, cell envelope-associated proteinases (CEPs) of lactic acid bacteria (LAB) may be able to produce hydrolysates that possess immunomodulatory activity. In this study, the selection of LAB by screening for bacterial CEP genes as well as by testing the proteolytic activity on bovine milk caseins was performed. Diverse hydrolytic ability against α_{S1}- and β-casein fractions was observed. Our experiments demonstrated that immunomodulatory activity of obtained casein hydrolysates (CHs) cannot be attributed to casein-derived bioactive peptides only and underlined the necessity to thoroughly remove bacterial components before assessing bioactivity of CHs. On this basis, this study established that 3 kDa-ultrafiltered CHs, produced after digestion with proteinases of *Lactobacillus acidophilus* ATCC 4356 and *Lactococcus lactis* subsp. *lactis* GR5, significantly decreased the basal nuclear factor (NF)-κB activity in Caco-2 cells, demonstrating immunomodulatory activity.

14th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 16-18 September, 2009 (Oristano, Italy)

Development of bioprocesses for the production of food protein hydrolysates containing bioactive peptides

Milda Stuknyte

The PhD thesis research project is aimed at development of bioprocesses for the production of food protein hydrolysates (FPHs) containing peptides with specific biological activities by exploiting the activity of Cell Envelope-associated Proteinases (CEP) of food-grade bacteria, in particular of lactic acid bacteria (LAB). The final object is the definition of completely food-grade bioprocesses, transferable to the industrial level and addressed to preparation of FPHs, which could be used as ingredients in functional foods.

15th Workshop on the Development in the Italian PhD Research on Food Science Technology and Biotechnology, 15-17 September, 2010 (Portici, Italy)

Development of bioprocesses for the production of food protein hydrolysates containing bioactive peptides

Milda Stuknyte

The first two activities of the PhD thesis project are described. Firstly, we performed the selection of lactic acid bacteria by screening bacterial cell envelope-associated proteinases (CEPs) at the genetic level as well as by testing the proteolytic activity on milk caseins. Moreover, fractionation of casein hydrolysates (CHs) by means of high-performance liquid chromatography (HPLC) was performed. Secondly, we developed a protocol for the *in vitro* biological analysis of CHs, addressed to determine their immunomodulatory activity by using a luciferase reporter gene system. This system is based on a nuclear factor kappa B (NF-κB)-inducible reporter plasmid, pNiFty2-Luc, in human colonic adenocarcinoma cells Caco-2.

10th Symposium on Lactic Acid Bacteria, 28 August – 1 September, 2011 (Egmond aan Zee, The Netherlands)

Lactic acid bacteria as potential probiotics for the pharyngeal mucosa

Guglielmetti S, Taverniti V, Minuzzo M, Arioli S, **Stuknyte M**, Zanoni I, Granucci F, Karp M, Mora D

The probiotic approach represents an alternative strategy in the prevention and treatment of infections in those body sites where the microbiota plays a role in the maintenance of physiological homeostasis. In this context, we evaluated in vitro the potential abilities of oral, probiotic and dairy bacteria in controlling *Streptococcus pyogenes* infections at the pharyngeal level. Initially, we analysed the bacterial adhesion on FaDu hypopharyngeal carcinoma cells and the ability to antagonize *S. pyogenes* on FaDu layer and HaCat keratinocytes. Due to their promising adhesive and antagonistic features, we studied the oral isolate *Streptococcus salivarius* ST3 and the dairy strain *Lactobacillus helveticus* MIMLh5 also through immunological in vitro experiments. We performed the quantification of several cytokines and the measurement of NF-κB activation in FaDu cells. Strain ST3 significantly increased NF-κB activation at baseline, while it had an opposite effect when FaDu cells were co-stimulated with IL-1β. In addition, ST3 biased cytokines production at baseline (reduction of IL-1β and TNFα, increase of MIP-1β and GM-CSF) and after IL-1β-induction (reduction on IL-6 and IL-8). MIMLh5 efficiently reduced the induction of IL-6, IL-8 and TNFα in a dose-dependent manner. After stimulating with IL-1β, active NF-κB was still markedly lowered. Nevertheless, we observed an increased secretion of IL-6, IFN-γ and GM-CSF under these conditions. In addition, MIMLh5 increased the GM-CSF/G-CSF ratio. This is compatible with a switch of the immune response towards a TH1 pathway, as supported by our observation that MIMLh5, once in contact with bone marrow derived dendritic cells, triggered the secretion of TNFα and IL-2. In conclusion, we propose ST3 and MIMLh5 as a potential probiotic bacteria for the human pharynx, with promising antagonistic and immunomodulatory properties.

16th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 21-23 September, 2011 (Lodi, Italy)

Immunomodulatory activity of bovine casein hydrolysates produced after digestion with proteinases of lactic acid bacteria

Milda Stuknytė

This report presents the results of the first PhD thesis topic, which dealt with the investigation of immunomodulatory activity of bovine casein hydrolysates (CHs), produced after digestion with proteinases of lactic acid bacteria. The study demonstrated that the 3 kDa-ultrafiltered CHs, produced after digestion with proteinases of *Lactobacillus acidophilus* ATCC 4356 and *Lactococcus lactis* subsp. *lactis* GR5, significantly decreased in vitro the basal NF-κB activity in intestinal epithelial-like Caco-2 cells, demonstrating immunomodulatory activity. In the case of *L. helveticus* MIMLh5, the immunomodulatory activity was likely due to the bacterial cell components present in the whole CH.

The 4th Interdepartmental Twinning Symposium, 28 September – 2 October, 2011 (Tammela, Finland)

Lactobacillus helveticus MIMLh5 S-layer protein-specific binders from antibody libraries

Stuknyte M, Brockmann E, Huovinen T, Taverniti V, Guglielmetti S, Mora D, De Noni I, Lamminmäki U

Many strains of *Lactobacillus helveticus* have been characterised as probiotics. These strains were reported to exert health benefits such as protection against infection, e.g. by modulating the immune system. *L. helveticus* MIMLh5, isolated from Grana Padano cheese natural whey starter, was proposed as a potential probiotic bacterium for the human pharynx with promising antagonistic and immunomodulatory properties (Guglielmetti et al., 2010). The surface layer (S-layer) protein was identified as being involved in the immunomodulatory properties of *L. helveticus* strains M92 (Beganović et al., 2011), MIMLh5 (Taverniti et al., manuscript in preparation).

Antibodies constitute a powerful tool to study protein function, protein localisation and protein-protein interactions, as well as for diagnostic and therapeutic purposes. Single-chain variable fragment antibodies (scFvs) have considerable potential for the immunological detection of small molecules, as well as proteins, in localisation of bacterial surface structures (Bradbury et al., 2011). In this study, a mix of two large human synthetic phage displayed libraries, protein-directed (Brockmann et al., 2011) and hapten-directed, was used to select scFvs against *L. helveticus* MIMLh5 S-layer protein. After three rounds of panning, four monoclonal scFvs capable of binding with the *L. helveticus* MIMLh5 S-layer protein and one capable of binding also with the S-layer protein of *L. helveticus* ATCC 15009, which is different only in five amino acids, were obtained. All five identified novel anti-S-layer scFvs were expressed in *Escherichia coli* XL1-Blue as alkaline phosphatase fusions (Krebber et al., 1997). ScFv-PolyH4 was used to develop an assay, based on Western blot analysis, for detection of the S-layer protein in Grana Padano cheese ripened for 2-5 months. These results showed promising applications of the scFv-PolyH4 for detecting immunologically important S-layer protein of *L. helveticus* in this cheese and in other food matrices and underlined the high value of the utilised phage display libraries for obtaining the extremely specific scFvs.

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