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Characterization of plant lectins for their ability to isolate *Mycobacterium avium* subsp. *paratuberculosis* from milk

Bernhard F. Hobmaier[a], Karina Lutterberg[a], Kristina J.H. Kleinwort[a], Ricarda Mayer[b], Sieglinde Hirmer[a], Barbara Amann[a], Christina Hölzel[b,c], Erwin P. Märtlbauer[b], Cornelia A. Deeg[a,*]

[a] Chair of Animal Physiology, Department of Veterinary Sciences, LMU Munich, Veterinärstraße 13, D-80539, Munich, Germany
[b] Chair of Hygiene and Technology of Milk, Department of Veterinary Sciences, LMU Munich, Schönleutnerstr 8, D-85764, Oberschleißheim, Germany
[c] Institute of Animal Breeding and Husbandry, Faculty of Agricultural and Nutritional Sciences, CAU Kiel, Hermann-Rodewald-Str. 6, 24098 Kiel, Germany

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* is the causative agent of paratuberculosis or Johne's disease, a chronic granulomatous enteritis in cattle and small ruminants, causing emaciation, decreased milk production and, in cattle, severe diarrhea (Arsenault et al., 2014). After infection, ruminants go through a long, asymptomatic subclinical phase, in which they cannot reliably be determined by standard diagnostic tests (Li et al., 2017). Apart from diseased animals, which can shed high numbers of MAP, animals in a subclinical stage of infection can also show intermittent shedding of MAP (Whitlock and Buergelt, 1996), which often remains undetected. Therefore, these subclinical animals play an important role in disease transmission and the contamination of milk, either by shedding MAP directly in milk (Sweeney et al., 1992) or by fecal contamination due to poor milking hygiene. Consumers can potentially be exposed to the pathogen through contaminated drinking-water and meat or dairy products obtained from infected ruminants (Gill et al., 2011). MAP, as well as other mycobacteria, has a very unique cell envelope, consisting of a plasma membrane, a thick cell wall, that contains large amounts of very long-chain fatty acids (mycolic acids), covalently bound to arabinogalactan and peptidoglycan, and an outermost layer. This cell envelope is a key element for the outstanding tenacity and drug-resistance of mycobacteria (Chiaradia et al., 2017). MAP was reported to survive many of the standard means of food decontamination (Chiodini and Hermon-Taylor, 1993; Grant et al., 2002). While the mycobacterial cell wall may play a role in this case, the specific mechanisms for its resistance to decontamination are still unclear (National Advisory Committee on Microbiological Criteria for Foods, 2010). More recently it was suggested that MAP in raw milk is primarily located inside somatic cells and thus physically protected by the large mass of the host cell (Gerrard et al., 2018). Viable MAP were found in pasteurized milk (Gerrard et al., 2018) and in dried dairy products like powdered infant formula (Botsaris et al., 2016) by using a bacteriophage amplification assay combined with PCR (phage-PCR). The zoonotic potential of MAP is still under discussion (Kuenstner et al., 2017). It could potentially play a role in inflammatory bowel diseases, like Crohn's disease and ulcerative colitis (Timms et al., 2016). Additionally, MAP infections could be involved in the pathogenesis of autoimmune diseases like type 1 diabetes, multiple sclerosis, rheumatoid arthritis and Hashimoto's thyroiditis (Garvey, 2018; Waddell et al., 2015). Although the causal link between MAP and these diseases is not proven, the possible association necessitates a reduction in human exposure through food to be implemented (Kuenstner et al., 2017). Therefore, a reliable, sensitive and time-saving method for the detection of MAP in food is vital. Especially for milk it is important that the detection method has the ability to differentiate viable MAP from non-viable MAP, which either entered the food chain already dead or were subsequently killed during pasteurization. The current gold standard for MAP detection is by cultivation (Slana et al., 2008), but when it comes to the detection of MAP in food, the method is limited, as it can only be deemed negative after weeks of incubation and still cannot guarantee negative status due to pre-culturing decontamination protocol (Sweeney et al., 2012). With faster methods, like PCR and qPCR one is able to detect small amounts of MAP DNA (Schonenbrucher et al., 2008; Soumya et al., 2009). However, PCR cannot distinguish viable from non-viable bacteria (Slana et al., 2008). Commonly used loci for PCR like IS900 and ISMap2 are known to share similar sequences with non-MAP mycobacteria, leading to the risk of false positive reactions if primers are not carefully designed (Englund et al., 2002; Park et al., 2018). Foddai and Grant (2017) described a phage assay as a fast method for the detection of living MAP in milk, but it must be combined with other methods (like magnetic separation or PCR), in order to achieve higher specificity (Foddai and Grant, 2017). Since the matrix for detection is milk, additional difficulties occur. In order to prevent overgrowing of plates with non-MAP microorganisms, culture from milk has to be combined with chemical decontamination, which leads to a decreased viability of MAP and reduces sensitivity of this test (Gao et al., 2005; Grant et al.,

* Corresponding author.
E-mail address: Cornelia.Deeg@lmu.de (C.A. Deeg).

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2003). High levels of fat and calcium ions in milk have an inhibitory effect on PCR sensitivity (Bickley et al., 1996; Lantz et al., 1994). For a sensitive and specific detection of MAP in milk, it is necessary to apply a specific separation and enrichment step prior to the final specific detection, in order to separate the MAP cells from the milk components. Specific separation not only gives the opportunity to remove inhibitory substances and non-target bacteria but also leads to highest possible concentrations of MAP in the sample. This enrichment of MAP, combined with simultaneous removal of inhibitory substances, leads to improved sensitivity and lower detection levels for the following detection method. Different approaches for the development of such methods were published, using either antibody or peptide coated paramagnetic beads (Foddai et al., 2010; Foddai and Grant, 2017; Husakova et al., 2017; O'Brien et al., 2016). In contrast to antibodies or MAP-binding peptides, plant lectins bind to specific glycoconjugates on the surface of MAP. It was recently shown that mannose binding lectin (MBL) is able to bind purified lipoarabinomannan (LAM) from *Mycobacterium tuberculosis* (H37Rv) *in vitro* (Seiler et al., 2018). Mannosylated lipoarabinomannan (ManLAM) is present in the cell walls of several pathogenic mycobacteria, including MAP (Turner and Torrelles, 2018). It is an important virulence factor by reprogramming the immune response of the host, creating an anti-inflammatory environment (Mosaiab et al., 2018). The mannose residues on ManLam are a possible target for mannose specific plant lectins (Barre et al., 2001; Nakamura-Tsuruta et al., 2008). Therefore, the goal of this study was to identify lectins that detect MAP-specific glycosylations and could therefore be eligible for further development of a specific enrichment method of MAP from milk samples.

2. Material and methods

2.1. Bacterial strains and milk

The bacterial strains used in the experiments were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). In detail, *Streptococcus thermophilus* (DSM-20617), *Streptococcus uberis* (DSM-20569), *Streptococcus dysgalactiae* (DSM, 20,666), *Lactococcus lactis* (DSM, 20,481), *Pseudomonas aeruginosa* (ATTC 9027), *Staphylococcus aureus* (ATTC 6538P), *Escherichia coli* (ATTC 3739), *Acinetobacter lwoffii* (DSM 2403), *Bacillus cereus* (DSM 4312), *Enterococcus faecium* (DSM 2918), *Enterococcus faecalis* (DSM 2570), *Enterococcus durans* (ATTC 13,755), *Mycobacterium intracellulare* (DSM 43,223), *Mycobacterium terrae* (DSM 43,227), *Mycobacterium scrofulaceum* (DSM 43,992), *Mycobacterium avium* subsp. *avium* (DSM 44,156), *Mycobacterium avium* subsp. *silvaticum* (DSM44175), *Mycobacterium asiaticum* (DSM 44,297) and *Mycobacterium avium* subsp. *paratuberculosis* (DSM 44,135) were used. Cows whole milk (1L, 3.5% fat) and low-fat milk (1L, 1.5% fat) were bought from the local supermarket. No-fat-dry-milk was purchased from Applichem (Darmstadt, Germany).

2.2. Lectins

A total of 36 plant lectins was tested for their binding efficiency with MAP and possible unwanted reactions with milk components and other, frequently occurring bacteria in this environment. The biotinylated and fluorescein labeled lectins were purchased from Biozol (Eching, Germany) and Linaris (Dossenheim, Germany). The following biotinylated lectins were used: *Aleuria aurantia* lectin (AAL, 2 mg/ml), *Agaricus bisporus* lectin (ABL, 2 mg/ml), *Amaranthus caudatus* lectin (ACL, 2 mg/ml), *Bauhinia purpurea* lectin (BPL, 2 mg/ml), Concanavalin A (ConA, 2 mg/ml), *Dolichos biflorus* agglutinin (DBA, 2 mg/ml), *Datura stramonium* lectin (DSL, 1 mg/ml), *Erythrina cristagalli* lectin (ECL, 1 mg/ml), *Galanthus nivalis* lectin (GNL, 2 mg/ml), *Griffonia simplicifolia* lectin I (GSL-I, 2 mg/ml), *Griffonia simplicifolia* lectin I Isolectin B4 (GSL-I-B4, 1 mg/ml), *Griffonia simplicifolia* lectin II (GSL-II, 1 g/ml), *Hippeastrum hybrid* lectin (HHL, 2 mg/ml), Jacalin (JAC, 1 mg/ml), *Lens culinaris* agglutinin (LCA, 2 mg/ml), *Lycopersicon esculentum* (tomato) lectin (LEL, 1 mg/ml), *Lotus tetragonolobus* lectin (LTL, 2 mg/ml), *Maackia amurensis* lectin II (MAL-II, 1 mg/ml), *Maclura pomifera* lectin (MPL, 2 mg/ml), *Musa paradisiaca* (banana) lectin (BanLec, 2 mg/ml), *Narcissus pseudonarcissus* lectin (NPL, 2 mg/ml), *Phaseolus vulgaris* erythroagglutinin (PHA-E, 2 mg/ml), *Phaseolus vulgaris* leucoagglutinin (PHA-L, 2 mg/ml), Peanut agglutinin (PNA, 5 mg/ml), *Pisum sativum* agglutinin (PSA, 5 mg/ml), *Psophocarpus tetragonolobus* lectin (PTL, 2 mg/ml), *Ricinus communis* agglutinin I (RCA-I, 2 mg/ml), Soybean agglutinin (SBA, 2 mg/ml), *Sophora japonica* agglutinin (SJA, 2 mg/ml), *Sambuccus nigra* agglutinin (SNA, 2 mg/ml), Solanum tuberosum (potato) lectin (STL, 1 mg/ml), *Ulex europaeus* agglutinin I (UEA-I, 2 mg/ml), *Vicia villosa* lectin (VVA, 1 mg/ml), *Wisteria floribunda* lectin (WFL, 2 mg/ml), Wheat germ agglutinin (WGA, 2 mg/ml) and succinylated Wheat germ agglutinin (sWGA, 2 mg/ml). Additionally, fluorescein labeled Banana lectin (2 mg/ml), fluorescein labeled Concanavalin A (5 mg/ml) and fluorescein labeled *Lens culinaris* agglutinin (1 mg/ml) were used.

2.3. Sample preparation

Before coating to the plates for the direct binding assay, MAP, control bacteria and milk were lysed. Therefore, the samples were resuspended in lysis buffer (9 M Urea, 2 M Thiourea, 65 mM Dithioerythritol, 4% CHAPS). Cells were sonicated six times for 30 s to disrupt cell walls. Protein concentration was determined via Bradford protein assay (SERVA, Heidelberg, Germany).

2.4. Binding efficiency to MAP

For the comparison of the binding efficiency of the 36 lectins to MAP, direct binding assays were performed. Microtiter plates (Maxisorp, Thermo Fisher Scientific, Bremen, Germany) were coated with lysed MAP diluted to 5 µg/ml in carbonate coating buffer, pH 9.6. The plates were refrigerated overnight to facilitate antigen binding. After blocking with polyvinylpyrrolidone (PVP; Sigma-Aldrich, Taufkirchen, Germany) dissolved in PBS-T buffer (pH 7.2) at 1% (w/v) for one hour at 37 °C, endogenous biotin was blocked with the streptavidin/biotin blocking kit (Biozol, Eching Germany). Subsequently, biotinylated lectins were added in a concentration of 1 µg/ml in PBS-T. After two hours at 37 °C, binding was detected with horseradish peroxidase conjugated streptavidin (Cell Signaling, Frankfurt, Germany) diluted 1/50,000 (v/v) in PBS-T for 30 min at 37 °C, followed by development with tetramethylbenzidine-solution (TMB) (Applichem, Darmstadt, Germany). The color development was stopped with 1M sulphuric acid and absorbance was determined at 450 nm (Sunrise microplate reader, Tecan, Crailsheim, Germany). Obtained optical densities led to the assorting of lectins into four groups. Group 1 (OD $<$ 1) was named “low efficiency”, group 2 (OD $\geq$ 1.0 and $<$ 1.5) “medium efficiency”, group 3 (OD $\geq$ 1.5 and $<$ 2.0) “high efficiency” and group 4 (OD $\geq$ 2.0) “very high efficiency”. Lectins from the “low efficiency” group were eliminated from the study, while the lectins from the remaining groups were used in the subsequent experiments.

2.5. Binding to milk components

For the determination of adverse reactions with milk, duplicates of wells on an ELISA-plate were either coated with lysed MAP, whole milk (5 µg/ml), low-fat milk (5 µg/ml) or no-fat-dry-milk (Applichem GmbH, Darmstadt, Germany, 5 µg/ml) and subsequently incubated with the respective lectin (1 µg/ml). Assays were performed as described (2.4.). The optical densities of the wells that were previously coated with milk (= OD (*Sample*)), were divided by the results of the MAP-coated wells (= OD (*MAP*)). The resulting quotient was named relative binding ratio.

$$Binding\ ratio = \frac{OD(Sample)}{OD(MAP)}$$

Lectins with higher optical densities in milk-coated wells compared to MAP-coated wells (ratio ≥ 1.0) were considered to bind milk better than MAP and were therefore eliminated as MAP enrichment candidates. Only lectins with medium (ratio < 1.0 and ≥ 0.5) and weak binding to milk (ratio < 0.5) were used for further experiments (Fig. 5).

2.6. Cross-reactivity to control bacteria

Cross-reactivity of lectins to control bacteria was tested with further assays. Lysates of twelve control bacteria (*Bacillus cereus, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus thermophilus, Streptococcus uberis, Acinetobacter lwofii, Escherichia coli* and *Pseudomonas aeruginosa*) and six environmental mycobacteria (*Mycobacterium avium* subsp. *avium, Mycobacterium avium* subsp. *silvaticum, Mycobacterium asiaticum, Mycobacterium intracellulare, Mycobacterium scrofulaceum* and *Mycobacterium terrae*) were tested. They were diluted to 5 μg/ml in bicarbonate buffer, pH 9.6. Bacteria were used in the same concentration as MAP which was included in the assays as positive control. As mentioned in section 2.5., resulting optical densities were divided by the optical densities of the respective lectin with MAP and expressed as a relative binding ratio. Cross reactions with a relative binding ratio higher than one were classified as "strong cross reactions". Adverse reactions with binding ratios above 0.5 were named "minor cross reactions". Lectins that showed high (more than ten strong cross reactions) and medium cross-reactivity (more than five strong cross reactions) were eliminated from the study (Fig. 5).

2.7. Immune-fluorescence microscopy of lectin binding to whole MAP cells

Binding to whole MAP cells was determined by fluorescence microscopy. MAP cells were washed off from Herrold's egg yolk medium, washed two times and resuspended in PBS (pH 7.4). To 10^9 CFU/ml MAP, 1 μg of fluorescein-labeled lectin was added and incubated over night at room temperature with gentle agitation. After three times washing with 200 μl TBS, the MAP-suspension was given into a 24 well plate and the plate was viewed with a Leica DMi8 microscope (Leica Microsystems, Wetzlar, Germany) at 40× magnification and fluorescence intensity was analyzed with Leica Application Suite X (LASX) software, version 1.1.4.1 (Leica microsystems, Wetzlar, Germany).

2.8. Inhibition of binding by mannose

To analyze the mannose dependency of respective lectin binding, MAP were incubated in TBS, containing 20% (w/v) α-D-mannose (Sigma-Aldrich, Taufkirchen, Germany), instead of pure TBS (negative control). After washing, pre-incubated samples were resuspended in 200 μl TBS and given into a well on a 24 well plate adjacent to a non-inhibited sample and viewed at 40× magnification. Both samples (inhibited and non-inhibited) were compared under identical settings for the depiction of fluorescence intensity.

3. Results

3.1. Four lectins bound to MAP very effectively

Thirty-six plant lectins were tested for their ability to bind to MAP cell surface glycosylations. The lectins were ranked according to their achieved optical densities and subsequently divided into four groups: "low efficiency", "medium efficiency", "high efficiency" and "very high efficiency" of MAP binding (Table 1). Twenty of the 36 lectins did not bind well to MAP (OD < 1) and were allocated to the "low efficiency" group: ABL, BPL, DBA, DSL, GNL, GSL-I, GSL-I-B4, GSL-II, HHL, MAL-II, MPL, NPL, PSA, SJA, SNA, STL, UEA-I, VVA, sWGA and WFL. The "medium efficiency" (OD ≥ 1.0 and < 1.5) group contained six lectins (JAC, PHA-E, PHA-L, PNA, PTL and WGA). Another six lectins (AAL, ACL, ECL, LTL, RCA-I and SBA) formed the "high efficiency" (OD ≥ 1.5 and < 2.0) group. Four lectins, namely *Lens culinaris* agglutinin (LCA), *Lycopersicon esculentum* lectin (LEL), *Musa paradisiaca* lectin (BanLec) and Concanavalin A (ConA) composed the group with very high efficiency (OD ≥ 2). Table 1 shows the 36 tested lectins ranked by their efficiency of binding MAP and their carbohydrate specificities. The twenty lectins from the "low efficiency" group were eliminated from further studies. Lectins from the "medium", "high" and "very high efficiency" groups could possibly be used for MAP enrichment because of their binding intensity (see Table 1).

3.2. Unwanted binding of lectins to milk components

As the goal of this study was identification of a lectin suited to separate MAP from milk, it was important to exclude candidates with substantial binding to milk components. Therefore, the 16 lectins that remained after elimination of the "low efficiency" group (Fig. 5), were further tested to subsequently exclude those with strong adverse reactions to milk components. In general, none of the lectins showed considerable differences in its reaction with different kinds of milk, except for RCA-I which showed increased binding with decreasing fat content of the milk products. The lectins JAC, RCA-I, ECL and AAL showed strong reactions with milk (ratio ≥ 1) which therefore led to exclusion from enrichment candidates for MAP from milk (Fig. 1). LEL, LTL, WGA, ConA and ACL showed medium reactivity (ratio < 1.0 and ≥ 0.5), while PHA-E, PHA-L, PTL, SBA, LCA, BanLec and PNA showed no, or only weak (ratio < 0.5) adverse reactions with milk (Fig. 1). Those twelve lectins were selected for further experiments (Fig. 5).

3.3. Unspecific reactions with control bacteria that could interfere with detection in food

After elimination of lectins that bound MAP inefficiently and/or showed strong adverse reactions with milk components (ratio ≥ 1), twelve lectins remained (Fig. 5). To avoid non-specific reactions with other common bacteria occurring in milk, those twelve lectins were also tested for cross reactions with six environmental mycobacteria and twelve control bacteria that are frequently found in milk (causative agents of food infections, -intoxications and mastitis plus several bacteria of gut and skin microbiota). LTL was the most cross-reactive of the tested lectins, with strong cross reactions (ratio ≥ 1) with 16 of the 18 tested control bacteria (Fig. 2, panel A). PNA and AAL also expressed strong cross reactions with a variety of the control bacteria (Fig. 2, panels B and C). Cross-reactivity of PTL and LEL was almost equal, with eight major and five minor (ratio ≥ 0.5) cross reactions (panels D and E). PHA-E and PHA-L (panels F and G) showed six strong and several weaker cross reactions (five for PHA-E and eight for PHA-L); therefore, those seven lectins were subsequently eliminated from the candidates. LCA, SBA and ACL predominantly cross-reacted with *Mycobacterium avium* subsp. *silvaticum* and *Pseudomonas aeruginosa* (Fig. 2, panels H–J). Additionally, LCA and SBA expressed minor cross reactions with five (SBA) or six (LCA) control bacteria. Although ACL's cross-reactivity was weaker than LCA's and SBA's, it was still more pronounced than those of BanLec and ConA, which were the strongest binding lectins. ConA cross-reacted with most of the tested *Mycobacterium* spp. except for *Mycobacterium terrae*, but with none of the other tested bacteria (Fig. 2, panel K). BanLec only cross-reacted with *Mycobacterium avium* subsp. *silvaticum*, *Mycobacterium asiaticum*, and *Pseudomonas aeruginosa*. Mild cross reactions were observed to *Mycobacterium avium* subsp. *avium* (Fig. 2, panel L). This made ConA and BanLec not only the lectins with the highest affinity for MAP, but also the most specific and therefore

Table 1
Evaluation of binding efficiency of tested lectins with MAP. Lectins were then sorted by their binding efficiency with MAP and categorized into four different groups. "low efficiency" (*), "medium efficiency" (**) "high efficiency" (***) and "very high efficiency" (****). The four lectins from the "very high efficiency"-group (*Lens culinaris* agglutinin, *Lycopersicon esculentum* lectin, *Musa paradisiaca* lectin, and Concanavalin A) could be possible candidates for MAP-enrichment.

Acronyme	Name	Binding to MAP	Specificity (manufactorer's specifications)
MAL-II	*Maackia amurensis* lectin II	–	Sialic acid in a α-2,3 linkage
PSA	*Pisum sativum* agglutinin	–	α-linked mannose oligosaccharides with an *N*-acetylchitobiose linked α-fucose residue included in the receptor sequence
BPL	*Bauhinia purpurea* lectin	*	Gycoconjugates containing galactosyl (β-1,3) *N*-acetylgalactosamine structures or terminal α-linked *N*-acetylgalactosamine
DBA	*Dolichos biflorus* agglutinin	*	α-linked *N*-acetylgalactosamine
STL	*Solanum tuberosum* lectin	*	*N*-acetylglucosamine-oligomers and oligosaccharides containing *N*-acetylglucosamine and *N*-acetylmuramic acid
GSL-II	*Griffonia simplicifolia* lectin II	*	α- or β-linked *N*-acetylglucosamine residues on the nonreducing terminal of oligosaccharides
SJA	*Sophora japonica* agglutinin	*	Terminal *N*-acetylgalactosamine and galactose residues (preferential binding to β anomers)
MPL	*Maclura pomifera* lectin	*	α-linked *N*-acetylgalactosamine structures
DSL	*Datura stramonium* lectin	*	(β-1,4) linked *N*-acetylglucosamine oligomers, preferring chitobiose or chitotriose
HHL	*Hippeastrum hybrid* lectin	*	(α-1,3) and (α-1,6) linked mannose structures
VVA	*Vicia villosa* lectin	*	Single α-*N*-acetylgalactosamine residues linked to serine or threonine in a polypeptide
WFA, WFL	*Wisteria floribunda* lectin	*	Carbohydrate structures terminating in *N*-acetylgalactosamine linked α or β to the 3 or 6 position of galactose
sWGA	succinylated Wheat germ agglutinin	*	Oligosaccharides containing terminal *N*-acetylglucosamine or chitobiose
GSL-I	*Griffonia simplicifolia* lectin I	*	α-*N*-acetylgalactosamine residues
ABL	*Agaricus bisporus* lectin	*	Galactosyl (β-1,3) *N*-acetylgalactosamine, Thomsen-Friedenreich antigen
GSL-I-B4	*Griffonia simplicifolia* lectin I Isolectin B4	*	α-galactose residues
SNA	*Sambuccus nigra* agglutinin	*	Sialic acid attached to terminal galactose in (α-2,6) and to a lesser degree, (α-2,3) linkage inhibited by lactose and galactose
GNL	*Galanthus nivalis* lectin	*	Structures containing (α-1,3) mannose residues
UEA-I	*Ulex europaeus* agglutinin I	*	α-linked fucose residues
NPL	*Narcissus pseudonarcissus* lectin	*	α-linked mannose, preferring polymannose structures containing (α-1,6) linkages
PTL	*Psophocarpus tetragonolobus* lectin	**	Carbohydrate structures containing α-linked *N*-acetylgalactosamine
PNA	Peanut agglutinin	**	Galactosyl (β-1,3) *N*-acetylgalactosamine structures (T-Antigen)
WGA	Wheat germ agglutinin	**	Oligosaccharides containing terminal *N*-acetylglucosamine or chitobiose
PHA-E	*Phaseolus vulgaris* erythroagglutinin	**	Terminal galactose, *N*-acetylglucosamine and mannose residues
PHA-L	*Phaseolus vulgaris* leucoagglutinin	**	*N*-Glykans; GlcNAc und (β1,6)-linked structures
JAC	Jacalin	**	Only O-glycosidically linked oligosaccharides, preferring galactosyl (β-1,3) *N*-acetylgalactosamine
ACL	*Amaranthus caudatus* lectin	***	Galactosyl (β-1,3) *N*-acetylgalactosamine structure
ECL	*Erythrina cristagalli* lectin	***	D-galactose and D-galactosides
SBA	Soybean agglutinin	***	Oligosaccharide structures with terminal α- or β-linked *N*-acetylgalactosamine or galactose residues
LTL	*Lotus tetragonolobus* lectin	***	α-linked L-fucose containing oligosaccharides
RCA-I	*Ricinus communis* agglutinin I	***	β–galactose residues, with a preference for terminal sugars, lactose is a potent inhibitor
AAL	*Aleuria aurantia* lectin	***	Fucose linked (α −1,6) to *N*-acetylglucosamine or fucose linked (α −1,3) to *N*-acetyllactosamine
LCA	*Lens culinaris* agglutinin	****	Sequences containing α-linked mannose residues; α-linked fucose residues attached to the *N*-acetylchitobiose portion of the core oligosaccharide markedly enhance affinity
LEL	*Lycopersicon esculentum* lectin	****	*N*-acetylglucosamine oligomers, preferrably trimers and tetramers
BanLec	*Musa paradisiaca* lectin	****	Internal α(1,3) glucosyl- and mannosyl- residues
ConA	Concanavalin A	****	α-linked mannose present as part of a "core oligosaccharide"

most suited for enrichment of MAP.

LCA, ConA and BanLec were the only three lectins that showed few unspecific reactions with milk and control bacteria and were simultaneously allocated in the group of lectins with "very high efficiency" in binding to MAP (see Table 2). Therefore, they were selected as the most promising candidates for the enrichment of MAP and used in further experiments as candidates for the enrichment of MAP from milk (Fig. 5). ACL and SBA also performed well in terms of adverse reactions with milk or control bacteria, but showed considerably lower binding efficiency than LCA, BanLec and ConA. It was also reported earlier that lactose has an inhibitory effect on the binding of SBA (Debray et al., 1981). Therefore, only LCA, BanLec and ConA were included in further experiments.

3.4. Lectin binding to whole MAP cells

For the development of a direct enrichment assay of MAP from milk, it is vital that the targeted structure of the lectins is accessible in whole MAP cells without further preparation of samples. To test if the antigen is on the cell surface and therefore directly accessible for the lectins, we next analyzed the binding of the three candidates (LCA, ConA and BanLec) to whole MAP cells in TBS. Respective lectins were positive in direct immunofluorescence, indicating their binding to whole MAP cells (Fig. 3). From the three lectins tested, ConA had the most intense fluorescent staining. Further, co-incubation of MAP with ConA led to an obvious agglutination of MAP cells into clusters (Fig. 3).

3.5. Inhibition of binding by mannose

To investigate if the binding of the three lectin candidates was mannose dependent, we tested their binding to MAP in presence of mannose. LCA, ConA and BanLec bound considerably weaker when pre-incubated in a medium with 20% (w/v) α-D-mannose (Fig. 4). Binding of LCA was almost completely inhibited by mannose, whereas in the samples with ConA and BanLec, there was still some binding detectable. Probably, ConA and BanLec prefer more complex saccharides than LCA since their binding to MAP could not be completely inhibited by mannose.

4. Discussion

While milk from single cows can sometimes contain high numbers of MAP, milk samples from industrial-scale milk production facilities typically present only small MAP amounts (Slana et al., 2009; Sweeney et al., 1992). In order to develop a fast and specific detection method for MAP in milk, it is helpful to concentrate those few MAP into a

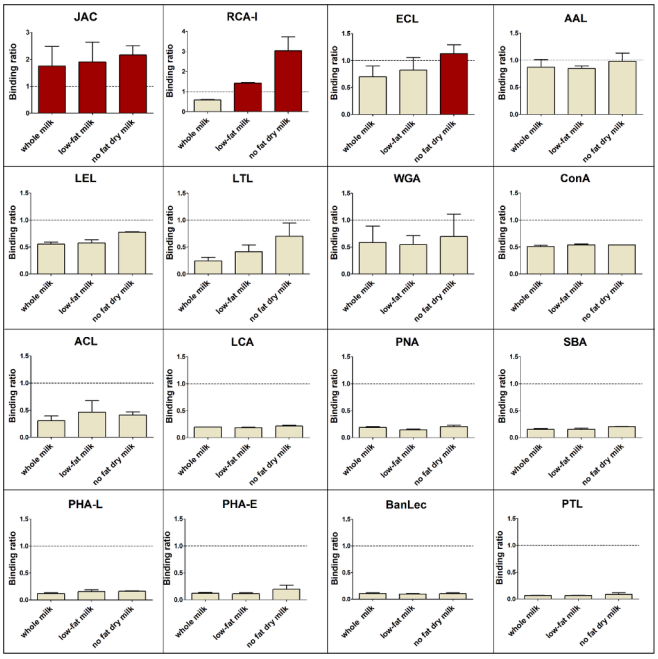

Fig. 1. Undesired binding of lectins to milk components: Reactivity with milk was determined via direct binding assay to MAP and different kinds of milk. The resulting optical densities were set in relation to the results obtained with MAP and displayed as a quotient (= relative binding ratio). JAC, RCA-I and ECL showed higher relative binding to milk than to MAP. AAL, LEL, LTL, WGA, ConA and ACL expressed moderate unspecific reactions, whereas PHA-E, PHA-L, PNA, SBA, LCA, BanLec and PTL only mildly reacted with milk.

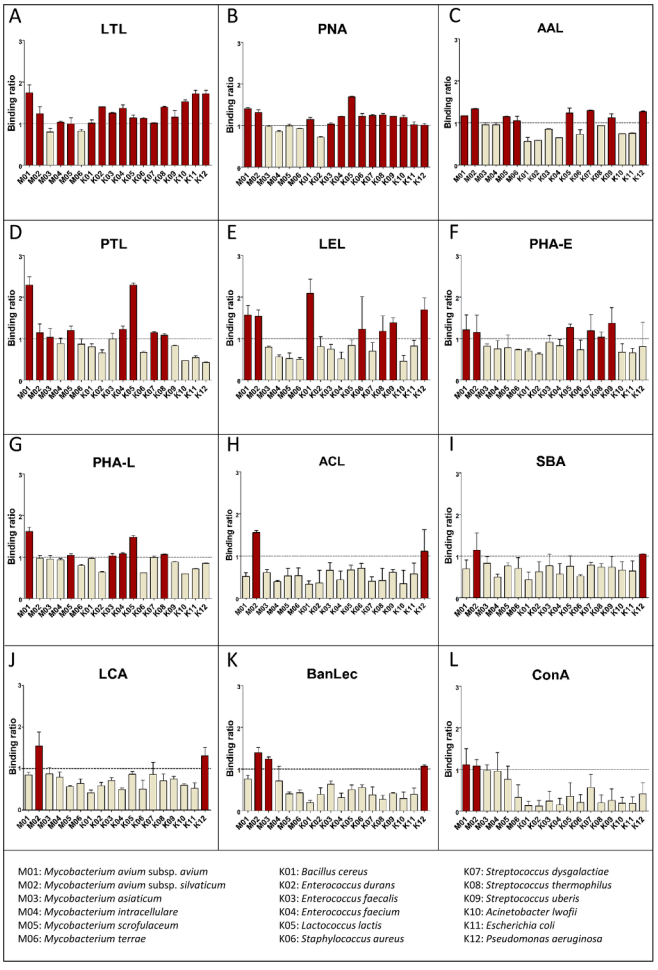

Fig. 2. Cross-reactivity of tested lectins with control bacteria: To determine possible cross reactions, the twelve remaining lectins were tested for their binding to MAP in comparison with six further *Mycobacterium* species and 12 bacteria, that are frequently found in milk. The resulting optical densities were set in relation to the results obtained with MAP and displayed as a quotient (= relative binding ratio). Bacteria that were bound more intense than MAP (ratio ≥ 1) were marked red. LTL (A) and PNA (B) cross-reacted with most of the control bacteria. AAL (C), PTL (D), LEL (E), PHA-E (F) and PHA-L (G) showed cross reactions with six or more of the control bacteria. ACL (H), SBA (I), LCA (J), BanLec (K) and ConA (L) had the least cross-reactivity. From the "very high efficiency group", only cross reactions of LCA, ConA and BanLec were weak or absent. Therefore, those lectins were used in further experiments.

smaller volume. Different methods for the separation and enrichment of MAP were published, using monoclonal or polyclonal antibodies or MAP-binding peptides (Foddai et al., 2010; Foddai and Grant, 2017; Husakova et al., 2017; O'Brien et al., 2016), but none is currently used in high-throughput commercial scale detection assays or replaced classical culture methods as the gold standard detection method. Lectins represent a different approach, targeting not proteins or peptides, but specific glycosylations on the cell wall of MAP. Lectins interact with carbohydrates non-covalently in a manner that is usually reversible and highly specific (Ambrosi et al., 2005). Their molecular size is much smaller than antibodies, allowing higher densities of carbohydrate sensing elements, leading to higher sensitivities and lower non-specific absorptions (Wang et al., 2012). Adding a lectin with an affinity for certain bacteria can also cause agglutination of respective bacteria into clusters (Raghu et al., 2017). In our study, we observed this agglutination after addition of ConA to whole MAP cells (Fig. 3). This clustering could potentially help in the enrichment, by making MAP more recoverable after centrifugation or by paramagnetic beads. In this study, we evaluated the suitability of plant lectins for the development of a specific pre-enrichment of MAP from milk. For this purpose, we analyzed the binding specificities of 36 plant lectins in regard to their binding efficiency to MAP and possible undesired reactions with milk or several control bacteria. In an initial screening assay, all 36 lectins were tested on MAP lysate. While investigating whole MAP cells in solution would probably have provided more detailed information about the binding of MAP to cell-surface structures, testing lysate gave us the opportunity to test as many lectins as possible in one single assay, enabling direct comparison of lectin results and minimizing inter-assay variation. Additionally, it allowed to test the binding of lectins to components of various control bacteria and semi-quantitative comparison of those results to MAP. This initial screening assay revealed very different binding efficiencies of the lectins to MAP. It was noticeable that particularly lectins with a mixed specificity for mannose and glucose (LCA, BanLec and ConA) or *N*-acetylglucosamine oligomers (LEL) bound with very high efficiency, while other mannose specific lectins that had no specificity for glucose showed far less efficiency in MAP binding (HHL, GNL and NPL). This suggests that the cell surface glycosylation of MAP might be a more complex structure than just free mannose residues. The lectins that formed the group with the highest efficiency for binding MAP (LCA, LEL, BanLec and ConA) were all reported to bind different parts of complex or oligomannose-type *N*-glycans (Debray et al., 1981; Oguri, 2005). For example, LEL recognizes different sugar chain units in complex-type and oligomannose-type *N*-glycans (Oguri, 2005). Its primary binding site in complex-type *N*-glycans is *N*-acetyllactosamine, while the chitobiose core appears to be the binding site in oligomannose-type *N*-glycans (Oguri, 2005). LEL binds to glycoproteins containing tri- or more highly branched complex-type *N*-glycans, but only weakly to bi-antennary complex-type *N*-glycans, indicating that the tri-antennary structure with the C-2,4 branch may be essential for binding of LEL (Oguri, 2005). In our experiments, LEL had the third highest MAP-binding efficiency. Its relative binding to milk was around 50% compared to MAP. However, when tested for cross

Table 2
Screening overview of all tests: After evaluating the binding properties and cross reactions of the lectins, *Lens culinaris* agglutinin (LCA), Concanavalin A (ConA) and *Musa paradisiaca* lectin (BanLec), were selected as possible candidates for a bead based enrichment of MAP.

Acronyme	Name	Binding to MAP	Cross-reactivity with milk	Cross-reactivity with control bacteria	Binding to whole MAP cells
MAL-II	*Maackia amurensis* lectin II	–	+++	+	n.a.
PSA	*Pisum sativum* agglutinin	–	++	+	n.a.
BPL	*Bauhinia purpurea* lectin	*	++	+	n.a.
MPL	*Maclura pomifera* lectin	*	++	–	n.a.
ABL	*Agaricus bisporus* lectin	*	+	+	n.a.
DSL	*Datura stramonium* lectin	*	+	–	n.a.
VVA	*Vicia villosa* lectin	*	+	–	n.a.
GSL-II	*Griffonia simplicifolia* lectin II	*	+/−	+	n.a.
sWGA	succinylated Wheat germ agglutinin	*	+/−	+	n.a.
WFL	*Wisteria floribunda* lectin	*	+/−	+	n.a.
NPL	*Narcissus pseudonarcissus* lectin	*	–	+	n.a.
STL	*Solanum tuberosum* lectin	*	–	+	n.a.
HHL	*Hippeastrum hybrid* lectin	*	–	+	n.a.
GSL-I-B4	*Griffonia simplicifolia* lectin I isolectin B4	*	–	+	n.a.
GNL	*Galanthus nivalis* lectin	*	–	+/−	n.a.
DBA	*Dolichos biflorus* agglutinin	*	–	–	n.a.
SJA	*Sophora japonica* agglutinin	*	–	–	n.a.
GSL-I	*Griffonia simplicifolia* lectin I	*	–	–	n.a.
UEA-I	*Ulex europaeus* agglutinin I	*	–	–	n.a.
JAC	Jacalin	**	+	+/−	n.a.
SNA	*Sambuccus nigra* agglutinin	**	+	–	n.a.
WGA	Wheat germ agglutinin	**	+/−	+/−	n.a.
PHA-E	*Phaseolus vulgaris* erythroagglutinin	**	–	+	n.a.
PTL	*Psophocarpus tetragonolobus* lectin	**	–	+/−	n.a.
PNA	Peanut agglutinin	**	–	+/−	n.a.
PHA-L	*Phaseolus vulgaris* leucoagglutinin	**	–	+/−	n.a.
ECL	*Erythrina cristagalli* lectin	***	+	+	n.a.
RCA-I	*Ricinus communis* agglutinin I	***	+	+	n.a.
AAL	*Aleuria aurantia* lectin	***	+/−	+	n.a.
LTL	*Lotus tetragonolobus* lectin	***	–	+	n.a.
ACL	*Amaranthus caudatus* lectin	***	–	–	n.a.
SBA	Soybean agglutinin	***	–	–	n.a.
LEL	*Lycopersicon esculentum* lectin	****	+/−	+	n.a.
ConA	Concanavalin A	****	+/−	–	+
LCA	*Lens culinaris* agglutinin	****	–	–	+
BanLec	*Musa paradisiaca* lectin	****	–	–	+

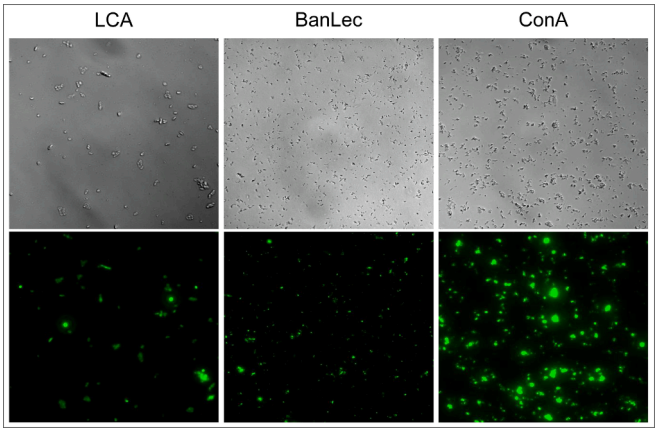

Fig. 3. Binding to whole MAP cells: To confirm binding under physiological conditions, whole MAP cells were incubated with fluorescein labeled *Lens culinaris* agglutinin (LCA), Concanavalin A (ConA) and *Musa paradisiaca* lectin (BanLec) (green). All three lectins were able to bind to the whole MAP cells.

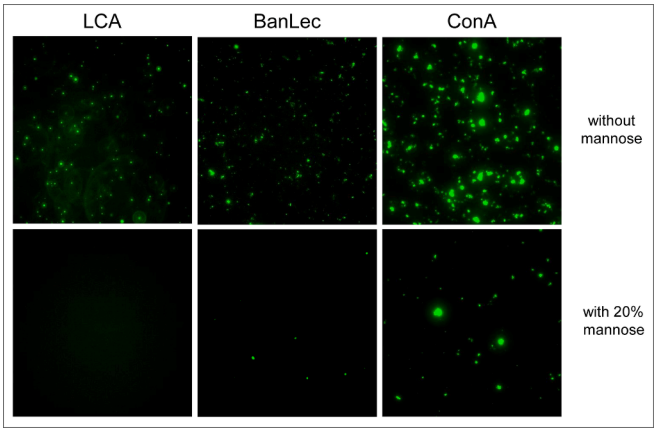

Fig. 4. Inhibition of lectin binding by mannose: To investigate the mannose dependency of the lectin binding, whole MAP cells were co-incubated with 20% (w/v) α-D-mannose and *Lens Culinaris* Agglutinin (LCA), Concanavalin A (ConA) and *Musa Paradisiaca* Lectin (BanLec) (green). Negative controls were only incubated with TBS. The binding of all three lectins was considerably inhibited by mannose.

reactions with other bacteria, it bound better to seven of the control bacteria (*Mycobacterium avium* subsp. *avium*, *Mycobacterium avium* subsp. *silvaticum*, *Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus thermophilus*, *Streptococcus uberis* and *Pseudomonas aeruginosa*) than to MAP. Another five bacteria (*Mycobacterium asiaticum*, *Enterococcus durans*, *Enterococcus faecalis*, *Lactococcus lactis* and *Escherichia coli*) were bound similar like MAP (ratio 0.75–1). This high cross-reactivity subsequently led to its exclusion, making LCA, ConA and BanLec the only remaining candidates. LCA and ConA are part of the legume lectin family (Damme et al., 1998). Both have great affinity to the core structure of complex-type *N*-glycans, which consists of a trimannosidic core substituted by two *N*-acetyl-β-glucosaminyl residues (Debray et al., 1981). Binding of LCA is greatly enhanced by the presence of an (α1-6)-linked fucose residue near the *N*-glycosidic linkage (Debray et al., 1981). LCA prefers bi-antennary *N*-acetyllactosaminic-type glycopeptides with galactose residues in a terminal non-reducing position, but

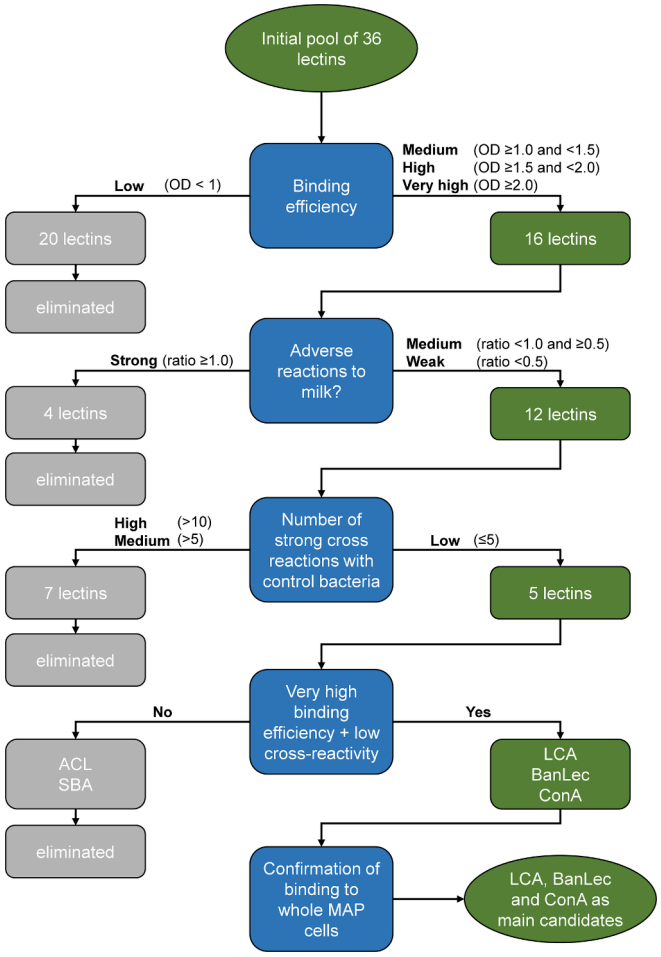

Fig. 5. Flow chart of the decision making process: After each screening experiment, lectins that did not meet the criteria were eliminated from the study (grey boxes), while the remaining lectins (green boxes) proceeded to the next experiment (blue boxes). From an initial pool of 36 lectins, 20 were removed after the first experiment due to low binding efficiency (OD < 1). The 16 remaining lectins were tested for unwanted reactions with milk. From this pool, the four lectins that showed strong adverse reactions to milk (ratio ≥ 1) were eliminated and the 12 lectins with medium (ratio < 1.0 and ≥ 0.5) or weak reactions (ratio < 0.5) proceeded to the next experiment. Through cross-reactivity tests with 12 control bacteria and six mycobacteria, we excluded seven lectins, showing cross-reactivity with more than five control bacteria. From the five remaining lectins, only those assorted to the "very high efficiency" group in the first experiment were selected as candidates.

shows very little affinity for saccharides with mannose residues in a terminal non-reducing position. In our study it showed fivefold increased binding to MAP as compared to milk (Fig. 1). LCA showed two strong and several weaker cross reactions with control bacteria. Cross reactions centered around the tested *Mycobacterium* spp., *Streptococcus* spp. and *Lactococcus lactis*. LCA's weaker binding to MAP, compared to BanLec and ConA, and the high cross-reactivity with frequent milk contaminant bacteria made it not suitable for an enrichment of MAP from milk. Therefore, only ConA and BanLec were left as candidates. ConA is one of the longest known plant lectins and was used for the immobilization of different glycoconjugates, due to its very broad specificity for mannose and glucose residues (Saleemuddin and Husain, 1991). Dobos et al. (1996) used ConA to identify different patterns of α-mannose residues in O-glycosylations of the 45 kDa glycoprotein of *Mycobacterium tuberculosis* (Dobos et al., 1996). ConA specifically bound with high affinity to oligosaccharides which contain the trimannose structure Man α1-6 (Man α1-3)Man (Ohyama et al., 1985). Unlike most other mannose specific lectins, ConA recognizes all three sugar units of the *N*-linked glycan trimannoside core (Naismith and Field, 1996). This explains why ConA has a significantly higher affinity for *N*-linked glycosides than for sole mannose residues. In contrast to LCA, the presence of an (α1-6)-linked mannose residue in a terminal non-reducing position (hybrid-type) and glycopeptides where the (α1-3) and the (α1-6)-linked mannose is substituted by four additional α-linked mannose residues (oligomannosidic-type structure) enhanced ConA's affinity (Debray et al., 1981), making its affinity for oligomannose-type and hybrid-type *N*-glycans higher than for the bi-antennary complex-type structure (Cummings et al., 2017). Unlike LCA, affinity of ConA does not depend on the substitution of the *N*-acetylglucosamine residues involved in the *N*-glycosidic linkage by an (α1-6)-linked fucose (Debray et al., 1981). Despite its rather broad specificity, ConA showed less cross-reactivity in our study than LCA. Only the two other species from the *Mycobacterium avium* complex (*Mycobacterium avium* subsp. *avium* and *silvaticum*) were bound more efficiently than MAP (ratio ≥ 1). Three other *Mycobacterium* species (*Mycobacterium asiaticum*, *intracellulare* and *scrofulaceum*) were bound with similar efficiency (ratio 0.75–1). This suggests that ConA recognizes similar or identical glycoproteins that are preserved throughout different *Mycobacterium* species, but not in the rest of the tested control bacteria. The reactivity of ConA with milk was similar to LEL, meaning that it binds MAP two times more efficiently than milk. Due to its outstanding binding efficiency to MAP and its low adherence to frequently occurring milk flora, it may be suitable for an enrichment of MAP. However, its sensitivity could be disturbed by unspecific reactions with milk. Additionally, we will perform further experiments to clarify if the observed agglutination of MAP after addition of ConA (Fig. 3), still occurs in milk and if this clustering helps in the separation of MAP from milk. Besides the big family of legume lectins, there is another lectin family with a specificity for *N*-glycans, the family of mannose binding Jacalin-related lectins (mJRLs) (Nakamura-Tsuruta et al., 2008). In this family BanLec forms a special subgroup, due to its very specific binding preferences (Nakamura-Tsuruta et al., 2008). It predominantly binds to longer (Man7-Man9) oligomannose-type glycans containing (α1-2)-linked mannose. BanLec has very high affinity for the Man-α1-2Man-α1-6Man-α1-6-Manβ structure of oligomannose-type glycans, but not to complex-type *N*-glycans (Nakamura-Tsuruta et al., 2008). Mannose-specific banana lectin (BanLec) is unique in its specificity for internal α1,3 linkages as well as β1,3 linkages at the reducing termini of oligosaccharides (Gavrovic-Jankulovic et al., 2008). Out of the possible candidates for a MAP enrichment, BanLec showed the least undesired reactions with milk in our study, binding MAP ten times better than milk. Except for cross-reactions with *Mycobacterium avium* subsp. *silvaticum, Mycobacterium asiaticum* and *Pseudomonas aeruginosa*, its overall cross-reactivity with control bacteria was very low. Its low binding to milk and milk related microorganisms makes it a good candidate for the development of a specific extraction method for MAP from milk. However, minimal non-specific enrichment of non-MAP bacteria is to be expected. Apart from plant lectins, human mannose binding lectin (MBL) was reported to bind isolated lipoarabinomannan from *Mycobacterium tuberculosis* in buffer, blood and urine (Seiler et al., 2018). The authors of respective study suggested that MBL could have high diagnostic potential, as LAM is an important target for point-of-care detection methods for tuberculosis (Iskandar et al., 2017; Lawn, 2012). These methods rely mainly on the detection of LAM in urine of patients via sandwich-ELISA. To our knowledge, binding of MBL to whole bacterial cells of *Mycobacterium tuberculosis* was not described yet. However, MBL was shown to bind to whole cells of *Mycobacterium avium* (Polotsky et al., 1997) and therefore likely also binds whole MAP cells. While urine represents a rather sterile environment with very few contaminants, milk contains a variety of non-target bacteria and other interfering substances. MBL, however, binds to a very broad spectrum of Gram-positive and Gram-negative bacteria, mycobacteria, viruses, fungi or pathogen associated molecular patterns (PAMPs) like Lipopolysaccharide endotoxin (LPS) or Lipoteichoic acid (LTA) (Seiler et al.,

2018). The very broad specificity of MBL for PAMPs makes it a formidable candidate for applications like diagnosis and treatment of sepsis (Cartwright et al., 2016; Kang et al., 2014). We did not test MBL in this study, as we focused on plant lectins and we expect MBL to show various cross reactions with different types of non-target bacteria in milk and milk itself. The macropattern-recognition of MBL relies on the three-dimensional orientation of several micropattern-recognition domains (Hoffmann et al., 1999). Those micropattern-recognition domains have a mixed affinity for glucose, fucose and mannose (Takahashi and Ezekowitz, 2005). In our study, we observed that lectins with a mixed mannose/glucose specificity (i.e. LCA, LEL, ConA and BanLec), achieved much higher binding efficiencies than lectins with a pure affinity for mannose residues. This indicates the presence of oligomannose and complex-type *N*-linked glycosylations on surface structures of MAP, therefore we will probably additionally screen MBL in the future. Both of them are recognized by ConA and LEL, while LCA only recognizes the complex-type and BanLec only the oligomannose-type *N*-glycans. The presence of *N*-linked glycosylations in MAP was already predicted on a genomic level (Rana et al., 2014), but to our knowledge, it was not verified on protein level to date. Our results confirm this prediction and they also prove presence of *N*-glycosylated glycoproteins on the outer cell membrane of MAP. A further specification of the glycan-structure on the cell membrane is given by the fact that LCA binds to MAP with high efficiency, whereas PSA does not. LCA and PSA are highly related legume lectins, with remarkable similarity in their binding preferences. Both share the affinity for branched complex-type *N*-glycans of the *N*-acetyllactosaminic type with a (α1-6)-linked fucose residue linked to the core oligosaccharide. However, their relative affinities for some glycopeptides are different: LCA prefers terminal *N*-acetylglucosamine residues in glycopeptides, whereas PSA has more affinity for terminal mannose residues that would decrease the affinity of LCA (Kornfeld et al., 1981). The high affinity of LCA and LEL together with the low affinity of PSA indicate the presence of branched complex-type *N*-glycans of the *N*-acetyllactosaminic (LacNAc) type, where the terminal LacNAc is not modified, resulting in a terminal non-reducing galactose residue. Our primary goal was to find lectins that are highly specific for MAP-exclusive glycoproteins. Although we did not find a lectin that exclusively binds to MAP, we found two lectins that bind to MAP with high efficiency and specificity. While ConA may cause some problems due to binding to milk components, BanLec does not seem to bind milk at all. Its narrow sugar-specificity and its low binding to milk components to milk make it a good choice for further investigation.

5. Conclusion

Plant lectins offer many advantages in the detection of pathogens, due to higher possible coating densities in consequence of their small size and to the quickly occurring agglutination between lectins and bacteria (Wang et al., 2012). Our results show, that lectins are able to bind MAP-cells with high efficiency and specificity, and that milk components are bound to a lesser extent. Therefore, we see great potential in the use of lectins as bioreceptors and will investigate in further studies whether they can be used for the development of a new enrichment method of MAP in milk.

Declaration of interest

None.

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