***In vitro* inhibition of *H. pylori* in a preferential manner using bioengineered *L. lactis* releasing guided Antimicrobial peptides**

Ankan Choudhury¹, Patrick Ortiz¹, Christopher M. Kearney¹

¹ Baylor University, Department of Biology, Waco, TX, USA

Correspondence:

Christopher M. Kearney

Chris_Kearney@baylor.edu

Abstract

Objectives: Targeted therapies seek to selectively eliminate a pathogen without disrupting the resident microbial community. This is even more important when a pathogen like *H. pylori* resides in stomach, a sensitive microbial ecosystem. Using a probiotic like *Lactococcus lactis* and bioengineering it to release a guided Antimicrobial Peptide (AMP) targeted towards the pathogen offers a pathway to specifically knock-out the deleterious species and not disturbing the stomach microbiome.

Results: Three AMPs, Alyteserin, CRAMP and Laterosporulin, were genetically fused to a guiding peptide MM1, which selectively binds to Vacuolating Toxin A (VacA) of *H. pylori* and cloned into an excretory vector pTKR inside *L. lactis*. When cultured together *in vitro*, the *L. lactis* bioengineered with guided AMPs selectively killed *H. pylori* when compared to *E. coli* or *Lactobacillus plantarum*, as determined by qPCR. Chemically synthesized Alyteserin and MM1-Alyteserin showed similar preferential inhibition of *H. pylori* when compared against *E. coli*, with the MIC of MM1-Alyteserin becoming significantly higher for *E. coli* than Alyteserin whereas no such effect was observed against *H. pylori*.

Conclusions: Probiotics bioengineered to excrete guided AMPs can be a novel and useful approach for combating pathogens without endangering the natural microbial flora. Given the wealth of AMPs and guiding ligands, both natural and synthetic, this approach can be adapted to develop a diverse array of chimeric guided AMPs and can be cloned into probiotics to create a safe and effective alternative to conventional chemical antibiotics.

Introduction

Helicobacter pylori is the source of one of the most prevalent infections in the world, with over 50% prevalence in many countries but often over 90% in Africa and East Asia (Salih, 2009). Over 60% of cases of gastric cancer can be attributed to *H. pylori* infection (Correa and Piazuelo, 2011), making it one of the most widespread cancers caused by an infectious agent (Wroblewski et al., 2010). Multidrug resistant strains of *H. pylori* constitute an increasing portion of *H. pylori* infections, from >10% in European countries to >40% of infections in Peru (Boyanova et al., 2019). Newest treatment regimens for *H. pylori* infection include triple and quadruple antibiotic therapies to match the growing challenge of antibiotic resistance. Such therapeutic regimens include combinations of amoxicillin, tetracycline, bismuth, metronidazole, clarithromycin, and more. In return, quadruple, quintuple, and sextuple antibiotic-resistant strains have been detected (Boyanova et al., 2019). This escalation of antibiotic resistance in *H. pylori* has heightened the need for new therapeutic strategies to combat infection. The multiple actions of these antibiotics such as rRNA inhibition, β -lactams, nucleic acid inhibitors also deleteriously effect off target bacteria, and a growing list of antibiotics administered to curb a single infection increases the dysbiosis of commensal microbiota caused by killing of off-target bacteria (Becattini et al., 2016; Langdon et al., 2016; Zarrinpar et al., 2018). Antibiotic-associated Dysbiosis often precipitates

into intestinal inflammatory diseases like colitis (Strati et al., 2021), worsens neuro-immune mechanisms and viscerosensory functionalities (Aguilera et al., 2015) and often makes way for bloom of pathogens (Vangay et al., 2015) creating other possibly more serious infectious diseases. This presents a dilemma, as stronger small molecule antibiotics are required to kill bacteria with ever-evolving antibiotic resistance mechanisms, but stronger antibiotics kill a wider variety of commensal bacteria (Becattini et al., 2016; Langdon et al., 2016; Zarrinpar et al., 2018).

To meet the challenges associated with this infection, one strategy proposed has been the use of AMPs. AMP refers to a broad group of short, usually cationic peptides with bactericidal or bacteriostatic properties (Lei et al., 2019). Because many of them exhibit a broad mechanism of action that forms pores in bacterial membranes, it has been suggested that it may be more difficult for bacteria to evolve resistance mechanisms to these peptides than traditional antibiotic drugs, though resistance can still occur (Assoni et al., 2020; Di et al., 2020; El Shazely et al., 2020). While the broad category of AMP comprises many diverse peptides that exhibit some antimicrobial activity, several specific types of AMP have been demonstrated to effectively kill *H. pylori*. Cathelicidins such as LL-37 and its murine homolog Cathelin-related Antimicrobial Peptide (CRAMP) have been demonstrated to effectively kill *Helicobacter pylori* in both *in vitro* and *in vivo* experiments (Hase et al., 2003; Zhang et al., 2016, 2013). Bacteriocins are small, stable AMPs released by other bacteria, that have broad bactericidal ability against a variety of gram-positive and gram-negative bacteria including *H. pylori* (Neshani et al., 2019). Among them, Type II bacteriocins including Laterosporulin has been well documented for their bactericidal activity with well-established mechanisms (Baindara et al., 2016; Singh et al., 2015). As more novel AMPs are discovered, a catalog of AMPs with activity against *H. pylori* has grown, showing promise as potential therapeutics.

While many of these AMPs have demonstrated effective antibacterial activity towards *H. pylori*, they also kill many other bacterial taxa. The double-edged sword of antibacterial therapies is that they have the unintended consequence of killing commensal microbiota. To deal with the problem of off target killing there have been several proposed solutions. Some AMPs naturally have increased activity towards specific bacterial taxa, and if utilized properly might avoid causing dysbiosis of microbiota in certain settings. Another option has been to modify AMPs, making chimeric peptides that use a short glycine linker and a guide peptide to “target” a specific taxon. Such guided antimicrobial peptides (gAMPs) have been shown to be effective in several settings against a variety of bacteria (Choudhury et al., 2020; Eckert et al., 2012, 2006; Kim et al., 2020). In some cases, such constructs can be made to increase the toxicity of a relatively weak AMP towards a targeted bacterium (Eckert et al., 2006), whereas in others it has been demonstrated to decrease toxicity of a potent AMP towards off-target bacteria (Choudhury et al., 2020). Furthermore, while studies have shown the bactericidal effects of such gAMPs in an *in-vivo* setting, the selectivity of these constructs has not been demonstrated *in-vivo* to ascertain if the microbiota are relatively undisturbed; nor has a gAMP been utilized against *H. pylori*. One of the reasons for this is that delivery of engineered peptides may be difficult. Intraperitoneal injections of purified peptide have been used for gut infections, but infections of the stomach require a delivery mechanism that will stand up to low pH conditions, peptidases, and provide delivery at the site of the infection. Antimicrobial peptides, being proteinaceous, are at a greater risk of enzymatic degradation through oral routes (Moncla et al., 2011; Svenson et al., 2008) and the high gastric acidity and peptidolytic enzymes cause breakdown of proteins and peptides when ingested orally. To avoid this gastric degradation, drugs are often delivered through systemic injection. For peptides, this is problematic as the size and high molecular weight of proteinaceous drug make it

an easier target for opsonization and neutralization by the blood complement system (Vaucher et al., 2011). Thus, for having the desired therapeutic effect the peptidic drug will have to survive the degradation in gut and reach the site of action. Encasing the antimicrobial peptide in a delivery system that masks it to survive the journey in the oral delivery and release it once the site is reached would be of great help and would help in microbial infections along the gut for which oral delivery of drugs is necessary.

Employing food grade bacterial systems like the lactic acid bacteria can solve the problem of the peptide's survival through degradative environments such as the gastrointestinal tract (Steidler et al., 2003). These bacteria are adapted to survive, propagate and produce and secrete their indigenous proteins in low pH conditions of the stomach. Encoding the chimeric antimicrobial peptide into a secretion vector inside such lactic acid bacteria will ensure that the protein will survive the journey into the gastrointestinal tract and be released from the cell into the site of infection (Jeong et al., 2006; Li et al., 2011). The cells will act as a sustained release platform as the expression of the protein will happen over a time. The cells will also replicate and maintain a colony of drug-releasing bacteria for an extended period (Drouault et al., 1999), unlike conventional drug delivery system. This reduces the number of dosages required to maintain the effective drug level for treatment of the infection. The vector can also be modified to contain an inducible promoter that is pH dependent (de Vos, 1999; Madsen et al., 1999), like the heat shock and nitrogen dependent constitutive promoters. A promoter that is induced by low acidic pH, like P1, P2 and P170 (de Vos, 1999; Madsen et al., 2005, 1999), will enable the lactic acid bacteria to express and secrete the encoded peptide only when it is exposed to such conditions at the target location in the gastric system. Thus, a lactic acid bacterium containing a secretion vector with a pH inducible promoter driving AMP expression constitutes an excellent sustained release drug

delivery system that will protect the peptidic drug from the enzymatic degradation in the gastrointestinal tract and deliver it to the proper target site. Lactic acid bacteria in the genera *Lactobacillus* and *Lactococcus* have been a part of the human diet for millennia. There have been numerous strains of lactic acid bacteria that are considered safe to consume and graded by the FDA as such (Nutrition, 2020). Since early 2000s, *Lactococcus lactis* has proven to be an excellent method for delivering engineered peptides *in-situ* from what has since burgeoned a variety of engineered probiotic bacteria. These engineered probiotics have been used to deliver signaling peptides, dyes, interleukins, and even unmodified AMPs (Foligne et al., 2007; Steidler et al., 2003, 1998). *Lactococcus lactis* also holds the distinction of being the first genetically engineered organism to be used for a therapeutic application in humans (Baat et al., 2006).

In this paper, we will demonstrate the efficacy of *L. lactis* bioengineered to secrete AMPs and gAMPs to inhibit *H. pylori* when co-cultured *in vitro*. We selected a suitable vector for such purpose and then cloned it with 3 AMPs of our choice and their corresponding gAMPs. The gAMPs were created by fusing a guiding peptide to the N- terminus of the corresponding AMP. The principle behind our gAMPs was to find a unique and attractive target protein on the surface of *H. pylori*, for which we picked Vacuolating cytotoxin A (VacA). This protein expressed by *H. pylori* is a major cause for its pathogenicity and can either be released into extracellular space (Cover and Blaser, 1992; Foegeding et al., 2016; Snider et al., 2016) or a significant portion of it is left in the bacterial cell surface (Foegeding et al., 2016; McClain and Cover, 2006; Telford et al., 1994; Voss et al., 2014) which is then transferred to host cell via contact dependent mechanisms (Ilver et al., 2004; Keenan et al., 2000; McClain and Cover, 2006). VacA toxin (comprised of the p33 and p55 subunits) binds to host cells and is internalized, causing severe “vacuolation” characterized by the accumulation of large vesicles that possess hallmarks of both late endosomes

and early lysosomes (Foegeding et al., 2016; Palframan et al., 2012). The development of “vacuoles” has been attributed to the formation of VacA anion-selective channels in membranes. Apart from its vacuolating effects, it has recently become clear that VacA also directly affects mitochondrial function (Foo et al., 2010). The VacA toxin binds to stomach lining cells by associating with the lipid rafts on the cell membrane which causes it to be internalized by the cell and promote vacuole formation (Fiocca et al., 1999). Once internalized, the p34 subunit of VacA toxin also forms an anionic pore into the mitochondrial membrane and interfere with its function (Domańska et al., 2010). The vacA toxin has been studied for being a major virulence factor and hence its structures and associations are also explored. One such protein whose association with the VacA toxin was explored was Multimerin-1 (Sato et al., 2013). Multimerin-1 is a protein expressed on the surface of human platelets and is bound by the VacA toxin when it induces platelet CD62P expression⁷⁶. It was shown that VacA would bind with a specified region of multimerin-1 from AA 321-340 stronger than any other region, making it the purported region for attachment with the platelets (Sato et al., 2013). We used this peptide sequence, fused to the AMP, as a targeting domain against *H. pylori*. Expression will be driven by the low pH induced secretion vector in *L. lactis*. This will yield a live vector delivery system for AMP targeted towards only *H. pylori* and sparing other bacteria.

For expressing the AMPs and gAMPs in *L. lactis* we picked the vector pT1NX (Steidler et al., 2004, 1998; van Asseldonk et al., 1990; Waterfield et al., 1995). pT1NX contains a native *L. lactis* promoter called P1 (Madsen et al., 2005) which is induced by the low pH conditions like in the stomach. P1 is also induced by thermal stress and have been seen to increase expression of proteins 500-fold when growth conditions are changed from 24°C to around 40°C, which is closer to a human physiological condition (de Vos, 1999). pT1NX also contains the native signal peptide

usp45 (van Asseldonk et al., 1990) that secretes any expressed protein placed downstream of it in the ORF. Thus, placing the AMP or the gAMP placed downstream in the ORF of the vector will ensure that the usp45 signal peptide will secrete it outside the *L. lactis* cell surface and this expression and subsequent secretion will be governed by the P1 promoter which will trigger expression only in low pH conditions. The pT1NX vector was further modified by adding a Kanamycin Resistant domain to make it a dual bacteria vector and making it possible to be cloned into *E. coli* along with *L. lactis*.

Methods and Materials

Cloning Antimicrobial peptides(AMPs) and guided Antimicrobial peptides (gAMPs) in L. lactis

The ORFs of the AMPs (Table 3.1), codon-optimized for *Lactococcus lactis* were cloned into the modified pT1NX plasmid (pT1NX-Kanamycin resistant/pTKR, Figure 3.1) in between the restriction enzyme sites BamHI and SpeI by replacing the spaX protein of the original plasmid. The P1 promoter upstream of the BamHI cut-site controls the downstream expression as a constitutive promoter which is upregulated by low pH. The usp45 gene immediately upstream of BamHI site is an endogenous signal peptide of *Lactococcus* species that transports the attached protein to extracellular location.

After ligation of the AMP/gAMP into pTKR vector, it was transformed into *E. coli* (10 β , NEB) and plated onto Kanamycin selective plate. The pT1NX plasmid (LMBP 3498) has erythromycin resistance but pTKR is a dual vector with kanamycin resistance for cloning into electrocompetent *E. coli* (10 β , NEB) for plasmid propagation. Extracted plasmid from the *E. coli* was then electroporated into electrocompetent *L. lactis* MG1363 (LMBP 3019) and plated on erythromycin selective GM17 plate (30°C, microaerobic, overnight). After screening for the

presence of the AMP/gAMP ORFs with PCR, selected colonies are propagated in liquid cultures of M17 broth with Glucose (0.5% w/v) in the presence of erythromycin (5 µg/ml).

AMP/ gAMP	Peptide Sequence
Laterosporulin	ACQCPDAISGWTHTDYQCHGLENKMYRHVYAICMNGTQVYCRTEWGSSC
Multimerin1(MM1)-Laterosporulin	MQKMTDQVNYQAMKL TLLQK SGGGS ACQCPDAISGWTHTDYQCHGLENKMYRHVYAICMNGTQVYCRTEWGSSC
Alyteserin	GLKDIFKAGLGSLVKGIAAHVAN
MM1-Alyteserin	MQKMTDQVNYQAMKL TLLQK SGGGS GLKDIFKAGLGSLVKGIAAHVAN
CRAMP	ISRLAGLLRKGGEKIGEKLLKKIGQKIKNFFQKLVPQPE
MM1-CRAMP	MQKMTDQVNYQAMKL TLLQK SGGGS ISRLAGLLRKGGEKIGEKLLKKIGQKIKNFFQKLVPQPE
Blue = Multimerin1, Red = linker	

TABLE 1. The peptide sequences of 3 AMPs and their corresponding gAMPs with Multimerin1 fragment fused to the N-terminus separated by a linker

FIGURE 1. pTKR (pT1NX with kanamycin resistance gene) vector with the AMP sequence (A) and the MM1-AMP/ gAMP sequence (B) in between the BamHI and SpeI cutsites downstream of P1 promoter and usp45 sequence.

In vitro assay: Co-culture assays with cloned *L. lactis* and *H. pylori* SS1

Desired variant of *L. lactis* cloned with AMP/tAMP were propagated from glycerol stocks and grown in GM17 broth overnight with erythromycin (5 µg/ml) with no shaking. *H. pylori* SS1 stocks were first propagated on Blood-Tryptic Soy agar (TSA) overnight with microaerobic condition and >5% CO₂ environment. Then colonies from the plate were transferred to a TS broth with 5% Newborn Calf serum (NCS) and grown overnight under microaerobic condition and >5% CO₂ environment. The *L. lactis* cultures were serially diluted in a 96 well culture plate with TS broth to make up a volume of 100 µL. To each well, 10 µL of the overnight *H. pylori* culture were added and each well volume was brought up to 200 µL with more TS broth. The plate is left to grow overnight in a microaerobic environment with >5% CO₂. After 24 hrs, the each well content from the culture plate is transferred on a 96 well PCR plate. That PCR plate was sealed and heated for 15 min at 100°C and chilled at 4°C for 5 min. Then the plate was centrifuged at 2000g for 2 min and the supernatant was used as the template for qPCR. The qPCR was done using primers for VacA gene to quantify *H. pylori* (forward: 5'-ATGGAAATACAACAAACACAC-3', reverse: 5'-CTGCTTGAATGCGCCAAAC-3') and primers for *acma* gene for quantifying *L. lactis* (forward: 5' GGAGCTCGTGAAAGCTGACT 3', reverse: 5' GCCGGAACATTGACAACCAC 3'). Standard curves for *H. pylori* and *L. lactis* were constructed by determining C_T values for different dilutions of the overnight cultures of the respective bacteria (1/10, 1/100, 1/1000, 1/10000) in the qPCR plates, the CFUs for the dilutions were determined by plating on their respective agar plates. Same procedure was followed by co-culturing *Lactobacillus plantarum* and *E. coli* with serially diluted cultures of *L. lactis* for 24 hrs and their amount was determined by qPCR using primers for species specific genes for either bacteria. The amount of *L. lactis* added to the co-cultures of all the three assays ranged from around 4000 to 512000 CFU/ul.

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229 *In vitro* assay of chemically synthesized AMP and gAMP against *H. pylori* and *E. coli*

230 The peptide Alyteserin (2.28 kD) and its guided counterpart MM1-Alyteserin (5.02 kD)
 231 were synthesized and purchased from GenScript USA. Aliquots of the AMP/gAMP were used to
 232 plate 96 well bactericidal assay using both *H. pylori* and *E. coli*. AMP/ gAMP were serially
 233 diluted to achieve a range of concentrations from 800 µM to 6.25µM in each wells. Each well
 234 had a total volume of 100 µl with 50 µl of peptide solution in PBS, 45 µl of growth media (TSB
 235 with 5% NCS for *H. pylori* and Lennox Broth for *E. coli*) and 5 µl of bacterial culture grown
 236 overnight and diluted to OD600 of 0.6. The assay plates for *H. pylori* were grown in
 237 microaerobic condition with >5% CO₂ at 37°C while *E. coli* plates were cultured in aerobic
 238 conditions overnight at 37°C. The MICs were determined by the concentration of the last well in
 239 ascending order that had bacterial growth associated turbidity. The inhibition of bacterial growth
 240 in the clear wells were confirmed by plating the clear well contents on a Mueller-Hinton agar at
 241 respective microaerobic and aerobic conditions.

242 **Results**

243 *L. lactis* cloned with gAMPs selectively killed *H. pylori* when co-cultured *in vitro*

244 On culturing together for 24 hrs, the cloned *L. lactis* showed a pronounced bactericidal
 245 activity towards *H. pylori* as determined by the qPCR method (Figure 2). The value of the *H. pylori*
 246 titer were calculated by using a concentration curve with Ct values found for CFU/ul values of *H.*
 247 *pylori* when plated. The gAMP containing *L. lactis* had similar bactericidal effect on *H. pylori* as
 248 their corresponding AMPs, except for MM1-Alyteserin showing a significant reduction of *H.*
 249 *pylori* CFUs at *L. lactis* doses around 64,000 CFU/ul or higher. The gAMP-*L. lactis*, at a

concentration of 64,000 CFU/ul of *L. lactis*, reduced the *H. pylori* load to 1/30th of the *H. pylori* load when co-cultured with *L. lactis* with empty vector at similar concentration on average, with MM1-Alyteserin-*L. lactis* reducing it to 1/1000th. The average reduction of *H. pylori* by the AMP-*L. lactis* was only 1/6th of that with the *L. lactis* containing empty vector, with Laterosporulin-*L. lactis* performing the best at 1/60th reduction at 64,000 CFU/ul. The reduction of *H. pylori* load increased with the concentration of *L. lactis* for both AMP and gAMP containing *L. lactis*.

FIGURE 2. The CFU/μl of *H. pylori* grown in the co-culture after 24 hrs of incubation, estimated from the corresponding C_t values determined by qPCR, plotted against the CFU/μl of *L. lactis* added to the co-culture

But when co-cultured with *L. plantarum* (Figure 3) and *E. coli* (Figure 4), the gAMP-*L. lactis* had significantly less bactericidal action on either bacterium compared to their corresponding AMP-*L. lactis*. The difference in reduction of both *E. coli* and *L. plantarum* was evident even at the lowest

concentration of *L. lactis* given (4000 CFU/ μ l). The bacterial load of *E. coli* and *L. plantarum* for the gAMP-*L. lactis* by 3.4x and 1.8x times higher on average respectively than the bacterial load when cultured with AMP-*L. lactis* at even 4000 CFU/ μ l, and the difference tapered with the increase in load of *L. lactis* upto 512000 CFU/ μ l. Hence, we can see that the *L. lactis* releasing gAMPs had significantly lesser killing potential for non-*H. pylori* bacteria than the *L. lactis* releasing corresponding AMPs, and this difference is only erased with exponentially higher doses of *L. lactis*. But inversely, gAMP releasing *L. lactis* killed *H. pylori* at almost similar rate with the AMP-*L. lactis* with MM1-Alyteserin performing the best among all the 6 AMP and gAMP tested.

FIGURE 3. The CFU/ μ l of *L. plantarum* found in the co-culture after 24 hrs of incubation, estimated from the corresponding C_t values determined by qPCR, plotted against the CFU/ μ l of *L. lactis* added to the co-culture

FIGURE 4. The CFU/ul of *E. coli* found in the co-culture after 24 hrs of incubation, estimated from the corresponding C_t values determined by qPCR, plotted against the CFU/ul of *L. lactis* added to the co-culture

Chemically synthesized gAMP showed preferential bactericidal activity towards *H. pylori* over *E. coli*

Bacterial inhibition assay with the chemically synthesized peptide demonstrated that Alyteserin when conjugated with the MM1 fragment domain had preferential inhibitory action against *H. pylori* over the non-target bacteria *E. coli*. The MIC recorded against *H. pylori* and *E. coli* for both Alyteserin and MM1-Alyteserin is shown in Table 2 and the mean values are shown graphically in Figure 5. The MIC against *H. pylori* for Alyteserin and MM1-Alyteserin were not significantly different even though MM1-Alyteserin had a lower MIC but the MIC against *E. coli* the MIC for MM1-Alyteserin was significantly higher ($p < 0.01$) than that of Alyteserin, thus showing that MM1-Alyteserin shows significantly lower bactericidal activity towards the non-target *E. coli* compared to *H. pylori*. This echoes the results seen when the *L. lactis* cloned with

AMPs and gAMPs were co-cultured with *H. pylori* and the non-target bacteria *E. coli* and *L. plantarum*.

Bacteria	MIC (μM)	Alyteserin	MM1-Alyteserin	P-value
<i>H. pylori</i>	Mean	83.8	61.2	0.1475
	SE	5.429	10.842	
<i>E. coli</i>	Mean	25.2	160.8	0.0004
	SE	1.906	11.80	

TABLE 2. Minimum Inhibitory Concentration (MIC) exhibited by chemically synthesized Alyteserin and MM1-Alyteserin against *H. pylori* and *E. coli*.

FIGURE 5. MIC values (μM) of Alyteserin and MM1-Alyteserin against *H. pylori* (A) and *E. coli* (B)

Discussion

Both the *in vitro* assays using *L. lactis* cloned with AMP/gAMP and using chemically synthesized Alyteserin/MM1- Alyteserin demonstrated effective inhibition of *H. pylori*. Co-culture assays showed that the *L. lactis* cloned with gAMPs that the Multimerin-1 fragment attached to it had similar efficacy against *H. pylori* to that of the *L. lactis* cloned with their AMP counterparts, with MM1-Alyteserin being the only gAMP that had significantly better microbicidal effect than its AMP counterpart. The measure of microbicidal effect was done by the estimating the CFU/ μ l of *H. pylori* in the co-culture after overnight incubation with a particular concentration of bioengineered *L. lactis*, the lesser *H. pylori* load the better microbicidal action exhibited. So, we can assume that attachment of MM1 fragment did not necessarily turn the AMP a more potential killer of the target bacteria, *H. pylori*. But when looking at the effect on the other two non-*Helicobacter* species; *E. coli* and *L. plantarum*, we see that the gAMPs with MM1 fragment had a significant drop in their microbicidal effect as a higher CFU/ μ l of gAMP-*L. lactis* were required to get a similar drop in non-target bacterial load exhibited by a lower CFU/ml dose of AMP-*L. lactis*. Hence, similar concentration of gAMP-*L. lactis* that caused a significant reduction in *H. pylori* CFU/ μ l did not have the same effect on either *E. coli* or *L. plantarum* unlike the AMP-*L. lactis* which had similar microbicidal effect on all three bacteria. This shows that the gAMPs released by the *L. lactis* did not kill the non-target bacteria as it would *H. pylori*, proving that it has achieved an extent of specificity towards *H. pylori*. The fragment of MM1 that we used was found to have the highest dissociation coefficient (K_D) with the vacA toxin on the surface of the *H. pylori* and may facilitate the attachment of the AMP to the bacteria. This enhanced attachment may not enhance the toxic effect that much as the amount of gAMP molecules approaching the bacterial cell membrane is not significantly different than the AMP molecules and attachment of

gAMP molecules to the membrane above the saturation concentration does not necessarily make it more toxic. But the MM1 fragment may be an impairment to the attachment of the gAMP molecules to the non-target bacterial cell membrane and hence decreasing attachment which translates to decreased toxicity. This effect has also been seen in previous literature where targeting moieties did not necessarily enhance the toxic activity but rather decreased action against the other non-target bacteria. This ensures that the gAMPs have a certain specificity towards the bacteria they are designed to kill and sparing other non-target species which may be beneficial to the host microbiome.

When using a chemically synthesized version of the peptide Alyteserin and MM1-Alyteserin too reflected the preferential microbicidal tendency when their MICs were determined against *H. pylori* and *E. coli*. Alyteserin is a class of AMPs among which Alyteserin-1 types show specific activity against Gram negative bacteria (Conlon et al., 2009). We used a specific sequence of an Alyteserin 1 peptide that has been used against Gram negative bacteria like *E. coli* (Conlon et al., 2009), *Salmonella* sp. (Volzing et al., 2013), *Acinetobacter* sp. (Conlon et al., 2010). The assay showed it to have similar MIC against *E. coli* as reported previously (~25 µM) but that MIC was increased significantly when using MM1-Alyteserin whereas there was no significant changes between the MICs of either Alyteserin and MM1 Alyteserin against *H. pylori*, albeit the MM1 Alyteserin having a lower MIC against *H. pylori* though it was not significant. This again proves the validity of our argument for using MM1 as a targeting domain and corroborates the data we got with co-culture assay using cloned *L. lactis* to inhibit *H. pylori* instead of synthesized peptides as the peptides have a danger of getting degraded when administered orally. And since both the naked peptide and the cloned *L. lactis* showed efficacy in killing *H. pylori* we can use the latter as

347 a model of therapeutic against the pathogen instead of the former and making it a more suited
348 candidate for oral administration.

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References

- A, V.R., C, V.G.C., Ap, C., V, S., J, F., A, B., 2011. Evaluation of the immunogenicity and in vivo toxicity of the antimicrobial peptide P34. *Int J Pharm* 421, 94–98. <https://doi.org/10.1016/j.ijpharm.2011.09.020>
- Aguilera, M., Cerdà-Cuellar, M., Martínez, V., 2015. Antibiotic-induced dysbiosis alters host-bacterial interactions and leads to colonic sensory and motor changes in mice. *Gut Microbes* 6, 10–23. <https://doi.org/10.4161/19490976.2014.990790>
- Assoni, L., Milani, B., Carvalho, M.R., Nepomuceno, L.N., Waz, N.T., Guerra, M.E.S., Converso, T.R., Darrieux, M., 2020. Resistance Mechanisms to Antimicrobial Peptides in Gram-Positive Bacteria. *Front. Microbiol.* 11. <https://doi.org/10.3389/fmicb.2020.593215>
- Axelsson, L., Ahrné, S., 2000. Lactic Acid Bacteria, in: Priest, F.G., Goodfellow, M. (Eds.), *Applied Microbial Systematics*. Springer Netherlands, Dordrecht, pp. 367–388. https://doi.org/10.1007/978-94-011-4020-1_13
- Baindara, P., Singh, N., Ranjan, M., Nallabelli, N., Chaudhry, V., Pathania, G.L., Sharma, N., Kumar, A., Patil, P.B., Korpole, S., 2016. Laterosporulin10: a novel defensin like Class IId bacteriocin from *Brevibacillus* sp. strain SKDU10 with inhibitory activity against microbial pathogens. *Microbiology (Reading)* 162, 1286–1299. <https://doi.org/10.1099/mic.0.000316>
- Becattini, S., Taur, Y., Pamer, E.G., 2016. Antibiotic-Induced Changes in the Intestinal Microbiota and Disease. *Trends Mol Med* 22, 458–478. <https://doi.org/10.1016/j.molmed.2016.04.003>
- Bermúdez-Humarán, L.G., Langella, P., Cortes-Perez, N.G., Gruss, A., Tamez-Guerra, R.S., Oliveira, S.C., Saucedo-Cardenas, O., Montes de Oca-Luna, R., Le Loir, Y., 2003. Intranasal immunization with recombinant *Lactococcus lactis* secreting murine interleukin-12 enhances antigen-specific Th1 cytokine production. *Infect Immun* 71, 1887–1896. <https://doi.org/10.1128/iai.71.4.1887-1896.2003>
- Bonning, B.C., Pal, N., Liu, S., Wang, Z., Sivakumar, S., Dixon, P.M., King, G.F., Miller, W.A., 2014. Toxin delivery by the coat protein of an aphid-vectored plant virus provides plant resistance to aphids. *Nature Biotechnology* 32, 102–105. <https://doi.org/10.1038/nbt.2753>
- Boyanova, L., Hadzhiyski, P., Kandilarov, N., Markovska, R., Mitov, I., 2019. Multidrug resistance in *Helicobacter pylori*: current state and future directions. *Expert Rev Clin Pharmacol* 12, 909–915. <https://doi.org/10.1080/17512433.2019.1654858>
- Braat, H., Rottiers, P., Hommes, D.W., Huyghebaert, N., Remaut, E., Remon, J.-P., van Deventer, S.J.H., Neirynck, S., Peppelenbosch, M.P., Steidler, L., 2006. A phase I

386 trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. Clin
387 Gastroenterol Hepatol 4, 754–759. <https://doi.org/10.1016/j.cgh.2006.03.028>

388 Buchholz, C.J., Friedel, T., Büning, H., 2015. Surface-Engineered Viral Vectors for
389 Selective and Cell Type-Specific Gene Delivery. Trends Biotechnol 33, 777–790.
390 <https://doi.org/10.1016/j.tibtech.2015.09.008>

391 Cao, J., de la Fuente-Nunez, C., Ou, R.W., Torres, M.D.T., Pande, S.G., Sinskey, A.J.,
392 Lu, T.K., 2018. Yeast-Based Synthetic Biology Platform for Antimicrobial
393 Peptide Production. ACS Synth Biol 7, 896–902.
394 <https://doi.org/10.1021/acssynbio.7b00396>

395 Chen, Y., Zhu, X., Zhang, X., Liu, B., Huang, L., 2010. Nanoparticles modified with
396 tumor-targeting scFv deliver siRNA and miRNA for cancer therapy. Mol Ther 18,
397 1650–1656. <https://doi.org/10.1038/mt.2010.136>

398 Choudhury, A., Islam, S.M.A., Ghidry, M.R., Kearney, C.M., 2020. Repurposing a drug
399 targeting peptide for targeting antimicrobial peptides against Staphylococcus.
400 Biotechnol Lett 42, 287–294. <https://doi.org/10.1007/s10529-019-02779-y>

401 Company, N., Nadal, A., La Paz, J.-L., Martínez, S., Rasche, S., Schillberg, S.,
402 Montesinos, E., Pla, M., 2014. The production of recombinant cationic α -helical
403 antimicrobial peptides in plant cells induces the formation of protein bodies
404 derived from the endoplasmic reticulum. Plant Biotechnol J 12, 81–92.
405 <https://doi.org/10.1111/pbi.12119>

406 Conlon, J.M., Ahmed, E., Pal, T., Sonnevend, A., 2010. Potent and rapid bactericidal
407 action of alyteserin-1c and its [E4K] analog against multidrug-resistant strains of
408 *Acinetobacter baumannii*. Peptides 31, 1806–1810.
409 <https://doi.org/10.1016/j.peptides.2010.06.032>

410 Conlon, J.M., Demandt, A., Nielsen, P.F., Leprince, J., Vaudry, H., Woodhams, D.C.,
411 2009. The alyteserins: two families of antimicrobial peptides from the skin
412 secretions of the midwife toad *Alytes obstetricans* (Alytidae). Peptides 30, 1069–
413 1073. <https://doi.org/10.1016/j.peptides.2009.03.004>

414 Correa, P., Piazzuelo, M.B., 2011. *Helicobacter pylori* Infection and Gastric
415 Adenocarcinoma. US Gastroenterol Hepatol Rev 7, 59–64.

416 Cover, T.L., Blaser, M.J., 1992. Purification and characterization of the vacuolating toxin
417 from *Helicobacter pylori*. J Biol Chem 267, 10570–10575.

418 Cripe, T.P., Wang, P.-Y., Marcato, P., Mahller, Y.Y., Lee, P.W., 2009. Targeting Cancer-
419 initiating Cells With Oncolytic Viruses. Mol Ther 17, 1677–1682.
420 <https://doi.org/10.1038/mt.2009.193>

421 de Vos, W.M., 1999. Gene expression systems for lactic acid bacteria. *Curr Opin*
422 *Microbiol* 2, 289–295. [https://doi.org/10.1016/S1369-5274\(99\)80050-2](https://doi.org/10.1016/S1369-5274(99)80050-2)

423 Devocelle, M., 2012. Targeted Antimicrobial Peptides. *Front. Immunol.* 3.
424 <https://doi.org/10.3389/fimmu.2012.00309>

425 Di, Y.P., Lin, Q., Chen, C., Montelaro, R.C., Doi, Y., Deslouches, B., 2020. Enhanced
426 therapeutic index of an antimicrobial peptide in mice by increasing safety and
427 activity against multidrug-resistant bacteria. *Science Advances* 6, eaay6817.
428 <https://doi.org/10.1126/sciadv.aay6817>

429 Domańska, G., Motz, C., Meinecke, M., Harsman, A., Papatheodorou, P., Reljic, B.,
430 Dian-Lothrop, E.A., Galmiche, A., Kepp, O., Becker, L., Günnewig, K., Wagner,
431 R., Rasso, J., 2010. *Helicobacter pylori* VacA toxin/subunit p34: targeting of an
432 anion channel to the inner mitochondrial membrane. *PLoS Pathog* 6, e1000878.
433 <https://doi.org/10.1371/journal.ppat.1000878>

434 Drouault, S., Corthier, G., Ehrlich, S.D., Renault, P., 1999. Survival, Physiology, and
435 Lysis of *Lactococcus lactis* in the Digestive Tract. *Appl Environ Microbiol* 65,
436 4881–4886.

437 Eckert, R., He, J., Yarbrough, D.K., Qi, F., Anderson, M.H., Shi, W., 2006a. Targeted
438 killing of *Streptococcus mutans* by a pheromone-guided “smart” antimicrobial
439 peptide. *Antimicrob. Agents Chemother.* 50, 3651–3657.
440 <https://doi.org/10.1128/AAC.00622-06>

441 Eckert, R., Qi, F., Yarbrough, D.K., He, J., Anderson, M.H., Shi, W., 2006b. Adding
442 Selectivity to Antimicrobial Peptides: Rational Design of a Multidomain Peptide
443 against *Pseudomonas* spp. *Antimicrob Agents Chemother* 50, 1480–1488.
444 <https://doi.org/10.1128/AAC.50.4.1480-1488.2006>

445 Eckert, R., Sullivan, R., Shi, W., 2012. Targeted Antimicrobial Treatment to Re-establish
446 a Healthy Microbial Flora for Long-term Protection. *Adv Dent Res* 24, 94–97.
447 <https://doi.org/10.1177/0022034512453725>

448 El Shazely, B., Yu, G., Johnston, P.R., Rolff, J., 2020. Resistance Evolution Against
449 Antimicrobial Peptides in *Staphylococcus aureus* Alters Pharmacodynamics
450 Beyond the MIC. *Front. Microbiol.* 11. <https://doi.org/10.3389/fmicb.2020.00103>

451 Ferreyra, J.A., Ng, K.M., Sonnenburg, J.L., 2014. The Enteric Two-Step: nutritional
452 strategies of bacterial pathogens within the gut. *Cell Microbiol* 16, 993–1003.
453 <https://doi.org/10.1111/cmi.12300>

454 Fiocca, R., Necchi, V., Sommi, P., Ricci, V., Telford, J., Cover, T.L., Solcia, E., 1999.
455 Release of *Helicobacter pylori* vacuolating cytotoxin by both a specific secretion
456 pathway and budding of outer membrane vesicles. Uptake of released toxin and

457 vesicles by gastric epithelium. *J Pathol* 188, 220–226.
 458 [https://doi.org/10.1002/\(SICI\)1096-9896\(199906\)188:2<220::AID-](https://doi.org/10.1002/(SICI)1096-9896(199906)188:2<220::AID-)
 459 [PATH307>3.0.CO;2-C](https://doi.org/10.1002/(SICI)1096-9896(199906)188:2<220::AID-PATH307>3.0.CO;2-C)

460 Foegeding, N.J., Caston, R.R., McClain, M.S., Ohi, M.D., Cover, T.L., 2016. An
 461 Overview of *Helicobacter pylori* VacA Toxin Biology. *Toxins* (Basel) 8.
 462 <https://doi.org/10.3390/toxins8060173>

463 Foligne, B., Dessein, R., Marceau, M., Poirer, S., Chamaillard, M., Pot, B., Simonet, M.,
 464 Daniel, C., 2007. Prevention and treatment of colitis with *Lactococcus lactis*
 465 secreting the immunomodulatory *Yersinia* LcrV protein. *Gastroenterology* 133,
 466 862–874. <https://doi.org/10.1053/j.gastro.2007.06.018>

467 Foo, J.H., Culvenor, J.G., Ferrero, R.L., Kwok, T., Lithgow, T., Gabriel, K., 2010. Both
 468 the p33 and p55 Subunits of the *Helicobacter pylori* VacA Toxin Are Targeted to
 469 Mammalian Mitochondria. *Journal of Molecular Biology* 401, 792–798.
 470 <https://doi.org/10.1016/j.jmb.2010.06.065>

471 Ganz, T., 2003. Defensins: antimicrobial peptides of innate immunity. *Nature Reviews*
 472 *Immunology* 3, 710–720. <https://doi.org/10.1038/nri1180>

473 Hancock, R.E., Diamond, G., 2000. The role of cationic antimicrobial peptides in innate
 474 host defences. *Trends Microbiol* 8, 402–410. <https://doi.org/10.1016/s0966->
 475 [842x\(00\)01823-0](https://doi.org/10.1016/s0966-842x(00)01823-0)

476 Hancock, R.E.W., Sahl, H.-G., 2006. Antimicrobial and host-defense peptides as new
 477 anti-infective therapeutic strategies. *Nature Biotechnology* 24, 1551–1557.
 478 <https://doi.org/10.1038/nbt1267>

479 Hase, K., Murakami, M., Iimura, M., Cole, S.P., Horibe, Y., Ohtake, T., Obonyo, M.,
 480 Gallo, R.L., Eckmann, L., Kagnoff, M.F., 2003. Expression of LL-37 by human
 481 gastric epithelial cells as a potential host defense mechanism against *Helicobacter*
 482 *pylori*. *Gastroenterology* 125, 1613–1625.
 483 <https://doi.org/10.1053/j.gastro.2003.08.028>

484 Holaskova, E., Galuszka, P., Frebort, I., Oz, M.T., 2015. Antimicrobial peptide
 485 production and plant-based expression systems for medical and agricultural
 486 biotechnology. *Biotechnology Advances, BioTech 2014 and 6th Czech-Swiss*
 487 *Biotechnology Symposium* 33, 1005–1023.
 488 <https://doi.org/10.1016/j.biotechadv.2015.03.007>

489 Ilver, D., Barone, S., Mercati, D., Lupetti, P., Telford, J.L., 2004. *Helicobacter pylori*
 490 toxin VacA is transferred to host cells via a novel contact-dependent mechanism.
 491 *Cell Microbiol* 6, 167–174. <https://doi.org/10.1046/j.1462-5822.2003.00349.x>

492 Institute of Medicine (US) Forum on Microbial Threats, 2010. Antibiotic Resistance:
493 Implications for Global Health and Novel Intervention Strategies: Workshop
494 Summary, The National Academies Collection: Reports funded by National
495 Institutes of Health. National Academies Press (US), Washington (DC).

496 Jeong, D.-W., Lee, J.-H., Kim, K.H., Lee, H.J., 2006. A food-grade expression/secretion
497 vector for *Lactococcus lactis* that uses an alpha-galactosidase gene as a selection
498 marker. *Food Microbiol* 23, 468–475. <https://doi.org/10.1016/j.fm.2005.06.003>

499 Kaplan, C.W., Sim, J.H., Shah, K.R., Kolesnikova-Kaplan, A., Shi, W., Eckert, R., 2011.
500 Selective membrane disruption: mode of action of C16G2, a specifically targeted
501 antimicrobial peptide. *Antimicrob Agents Chemother* 55, 3446–3452.
502 <https://doi.org/10.1128/AAC.00342-11>

503 Kau, A.L., Ahern, P.P., Griffin, N.W., Goodman, A.L., Gordon, J.I., 2011. Human
504 nutrition, the gut microbiome and the immune system. *Nature* 474, 327–336.
505 <https://doi.org/10.1038/nature10213>

506 Keenan, J., Day, T., Neal, S., Cook, B., Perez-Perez, G., Allardyce, R., Bagshaw, P.,
507 2000. A role for the bacterial outer membrane in the pathogenesis of *Helicobacter*
508 *pylori* infection. *FEMS Microbiol Lett* 182, 259–264.
509 <https://doi.org/10.1111/j.1574-6968.2000.tb08905.x>

510 Kim, H., Jang, J.H., Kim, S.C., Cho, J.H., 2020. Development of a novel hybrid
511 antimicrobial peptide for targeted killing of *Pseudomonas aeruginosa*. *European*
512 *Journal of Medicinal Chemistry* 185, 111814.
513 <https://doi.org/10.1016/j.ejmech.2019.111814>

514 Langdon, A., Crook, N., Dantas, G., 2016. The effects of antibiotics on the microbiome
515 throughout development and alternative approaches for therapeutic modulation.
516 *Genome Med* 8. <https://doi.org/10.1186/s13073-016-0294-z>

517 Lei, J., Sun, L., Huang, S., Zhu, C., Li, P., He, J., Mackey, V., Coy, D.H., He, Q., 2019.
518 The antimicrobial peptides and their potential clinical applications. *Am J Transl*
519 *Res* 11, 3919–3931.

520 Li, L., Guo, L., Lux, R., Eckert, R., Yarbrough, D., He, J., Anderson, M., Shi, W., 2010.
521 Targeted Antimicrobial Therapy Against *Streptococcus mutans* Establishes
522 Protective Non-cariogenic Oral Biofilms and Reduces Subsequent Infection. *Int J*
523 *Oral Sci* 2, 66–73. <https://doi.org/10.4248/IJOS10024>

524 Li, R., Takala, T.M., Qiao, M., Xu, H., Saris, P.E.J., 2011. Nisin-selectable food-grade
525 secretion vector for *Lactococcus lactis*. *Biotechnol Lett* 33, 797–803.
526 <https://doi.org/10.1007/s10529-010-0503-6>

527 Li, Y., 2011. Recombinant production of antimicrobial peptides in *Escherichia coli*: a
528 review. *Protein Expr Purif* 80, 260–267. <https://doi.org/10.1016/j.pep.2011.08.001>

529 Madsen, S.M., Arnau, J., Vrang, A., Givskov, M., Israelsen, H., 1999. Molecular
530 characterization of the pH-inducible and growth phase-dependent promoter P170
531 of *Lactococcus lactis*. *Mol Microbiol* 32, 75–87. <https://doi.org/10.1046/j.1365-2958.1999.01326.x>
532

533 Madsen, S.M., Hindré, T., Le Pennec, J.-P., Israelsen, H., Dufour, A., 2005. Two acid-
534 inducible promoters from *Lactococcus lactis* require the cis-acting ACiD-box and
535 the transcription regulator RcfB. *Mol Microbiol* 56, 735–746.
536 <https://doi.org/10.1111/j.1365-2958.2005.04572.x>

537 Malanovic, N., Lohner, K., 2016. Antimicrobial Peptides Targeting Gram-Positive
538 Bacteria. *Pharmaceuticals (Basel)* 9. <https://doi.org/10.3390/ph9030059>

539 Mannam, P., Jones, K.F., Geller, B.L., 2004. Mucosal vaccine made from live,
540 recombinant *Lactococcus lactis* protects mice against pharyngeal infection with
541 *Streptococcus pyogenes*. *Infect Immun* 72, 3444–3450.
542 <https://doi.org/10.1128/IAI.72.6.3444-3450.2004>

543 Marty, C., Schwendener, R.A., 2005. Cytotoxic tumor targeting with scFv antibody-
544 modified liposomes. *Methods Mol Med* 109, 389–402. <https://doi.org/10.1385/1-59259-862-5:389>
545

546 McClain, M.S., Cover, T.L., 2006. CHAPTER 24 - *Helicobacter pylori* vacuolating toxin,
547 in: Alouf, J.E., Popoff, M.R. (Eds.), *The Comprehensive Sourcebook of Bacterial*
548 *Protein Toxins* (Third Edition). Academic Press, London, pp. 468–490.
549 <https://doi.org/10.1016/B978-012088445-2/50029-9>

550 Moncla, B.J., Pryke, K., Rohan, L.C., Graebing, P.W., 2011. Degradation of naturally
551 occurring and engineered antimicrobial peptides by proteases. *Adv Biosci*
552 *Biotechnol* 2, 404–408. <https://doi.org/10.4236/abb.2011.26059>

553 Morassutti, C., De Amicis, F., Skerlavaj, B., Zanetti, M., Marchetti, S., 2002. Production
554 of a recombinant antimicrobial peptide in transgenic plants using a modified
555 VMA intein expression system. *FEBS Letters* 519, 141–146.
556 [https://doi.org/10.1016/S0014-5793\(02\)02741-2](https://doi.org/10.1016/S0014-5793(02)02741-2)

557 Mygind, P.H., Fischer, R.L., Schnorr, K.M., Hansen, M.T., Sönksen, C.P., Ludvigsen, S.,
558 Raventós, D., Buskov, S., Christensen, B., De Maria, L., Taboureau, O., Yaver,
559 D., Elvig-Jørgensen, S.G., Sørensen, M.V., Christensen, B.E., Kjærulff, S.,
560 Frimodt-Møller, N., Lehrer, R.I., Zasloff, M., Kristensen, H.-H., 2005. Plectasin
561 is a peptide antibiotic with therapeutic potential from a saprophytic fungus.
562 *Nature* 437, 975–980. <https://doi.org/10.1038/nature04051>

563 Neshani, A., Zare, H., Eidgahi, M.R.A., Chichaklu, A.H., Movaghar, A., Ghazvini, K.,
564 2019. Review of antimicrobial peptides with anti-*Helicobacter pylori* activity.
565 *Helicobacter* 24, e12555. <https://doi.org/10.1111/hel.12555>

566 Nicholson, J.K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., Pettersson, S.,
567 2012. Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267.
568 <https://doi.org/10.1126/science.1223813>

569 Norian, L.A., James, B.R., Griffith, T.S., 2011. Advances in Viral Vector-Based TRAIL
570 Gene Therapy for Cancer. *Cancers (Basel)* 3, 603–620.
571 <https://doi.org/10.3390/cancers3010603>

572 Nutrition, C. for F.S. and A., 2020. Microorganisms & Microbial-Derived Ingredients
573 Used in Food (Partial List) [WWW Document]. FDA. URL
574 [https://www.fda.gov/food/generally-recognized-safe-gras/microorganisms-](https://www.fda.gov/food/generally-recognized-safe-gras/microorganisms-microbial-derived-ingredients-used-food-partial-list)
575 [microbial-derived-ingredients-used-food-partial-list](https://www.fda.gov/food/generally-recognized-safe-gras/microorganisms-microbial-derived-ingredients-used-food-partial-list) (accessed 5.25.21).

576 Oeemig, J.S., Lynggaard, C., Knudsen, D.H., Hansen, F.T., Nørgaard, K.D., Schneider,
577 T., Vad, B.S., Sandvang, D.H., Nielsen, L.A., Neve, S., Kristensen, H.-H., Sahl,
578 H.-G., Otzen, D.E., Wimmer, R., 2012. Eurocin, a new fungal defensin: structure,
579 lipid binding, and its mode of action. *J Biol Chem* 287, 42361–42372.
580 <https://doi.org/10.1074/jbc.M112.382028>

581 Palframan, S.L., Kwok, T., Gabriel, K., 2012. Vacuolating cytotoxin A (VacA), a key
582 toxin for *Helicobacter pylori* pathogenesis. *Front Cell Infect Microbiol* 2.
583 <https://doi.org/10.3389/fcimb.2012.00092>

584 Peschen, D., Li, H.-P., Fischer, R., Kreuzaler, F., Liao, Y.-C., 2004. Fusion proteins
585 comprising a *Fusarium* -specific antibody linked to antifungal peptides protect
586 plants against a fungal pathogen. *Nature Biotechnology* 22, 732–738.
587 <https://doi.org/10.1038/nbt970>

588 Reinhardt, A., Neundorff, I., 2016. Design and Application of Antimicrobial Peptide
589 Conjugates. *Int J Mol Sci* 17. <https://doi.org/10.3390/ijms17050701>

590 Robinson, K., Chamberlain, L.M., Schofield, K.M., Wells, J.M., Le Page, R.W., 1997.
591 Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. *Nat*
592 *Biotechnol* 15, 653–657. <https://doi.org/10.1038/nbt0797-653>

593 Salih, B.A., 2009. *Helicobacter pylori* Infection in Developing Countries: The Burden for
594 How Long? *Saudi J Gastroenterol* 15, 201–207. [https://doi.org/10.4103/1319-](https://doi.org/10.4103/1319-3767.54743)
595 [3767.54743](https://doi.org/10.4103/1319-3767.54743)

596 Satoh, K., Hirayama, T., Takano, K., Suzuki-Inoue, K., Sato, T., Ohta, M., Nakagomi, J.,
597 Ozaki, Y., 2013. VacA, the vacuolating cytotoxin of *Helicobacter pylori*, binds to

598 multimerin 1 on human platelets. *Thromb J* 11, 23. <https://doi.org/10.1186/1477->
599 9560-11-23

600 Singh, P.K., Solanki, V., Sharma, S., Thakur, K.G., Krishnan, B., Korpole, S., 2015. The
601 intramolecular disulfide-stapled structure of laterosporulin, a class IId bacteriocin,
602 conceals a human defensin-like structural module. *FEBS J* 282, 203–214.
603 <https://doi.org/10.1111/febs.13129>

604 Snider, C.A., Voss, B.J., McDonald, W.H., Cover, T.L., 2016. Growth phase-dependent
605 composition of the *Helicobacter pylori* exoproteome. *J Proteomics* 130, 94–107.
606 <https://doi.org/10.1016/j.jprot.2015.08.025>

607 Steidler, L., Hans, W., Schotte, L., Neiryneck, S., Obermeier, F., Falk, W., Fiers, W.,
608 Remaut, E., 2000. Treatment of murine colitis by *Lactococcus lactis* secreting
609 interleukin-10. *Science* 289, 1352–1355.
610 <https://doi.org/10.1126/science.289.5483.1352>

611 Steidler, L., Neiryneck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B.,
612 Cox, E., Remon, J.P., Remaut, E., 2003. Biological containment of genetically
613 modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. *Nat*
614 *Biotechnol* 21, 785–789. <https://doi.org/10.1038/nbt840>

615 Steidler, L., REMAUT, R.E., Fiers, W., 2004. USE OF A CYTOKINE-PRODUCING
616 *LACTOCOCCUS* TRIBE FOR TREATING COLUMN. DE69914932T2.

617 Steidler, L., Robinson, K., Chamberlain, L., Schofield, K.M., Remaut, E., Le Page, R.W.,
618 Wells, J.M., 1998a. Mucosal delivery of murine interleukin-2 (IL-2) and IL-6 by
619 recombinant strains of *Lactococcus lactis* coexpressing antigen and cytokine.
620 *Infect Immun* 66, 3183–3189. <https://doi.org/10.1128/IAI.66.7.3183-3189.1998>

621 Steidler, L., Viaene, J., Fiers, W., Remaut, E., 1998b. Functional display of a
622 heterologous protein on the surface of *Lactococcus lactis* by means of the cell
623 wall anchor of *Staphylococcus aureus* protein A. *Appl Environ Microbiol* 64,
624 342–345. <https://doi.org/10.1128/AEM.64.1.342-345.1998>

625 Strati, F., Pujolassos, M., Burrello, C., Giuffrè, M.R., Lattanzi, G., Caprioli, F., Troisi, J.,
626 Facciotti, F., 2021. Antibiotic-associated dysbiosis affects the ability of the gut
627 microbiota to control intestinal inflammation upon fecal microbiota
628 transplantation in experimental colitis models. *Microbiome* 9, 39.
629 <https://doi.org/10.1186/s40168-020-00991-x>

630 Svenson, J., Stensen, W., Brandsdal, B.-O., Haug, B.E., Monrad, J., Svendsen, J.S.,
631 2008a. Antimicrobial peptides with stability toward tryptic degradation.
632 *Biochemistry* 47, 3777–3788. <https://doi.org/10.1021/bi7019904>

633 Svenson, J., Stensen, W., Brandsdal, B.-O., Haug, B.E., Monrad, J., Svendsen, J.S.,
634 2008b. Antimicrobial peptides with stability toward tryptic degradation.
635 Biochemistry 47, 3777–3788. <https://doi.org/10.1021/bi7019904>

636 Telford, J.L., Ghiara, P., Dell’Orco, M., Comanducci, M., Burroni, D., Bugnoli, M.,
637 Tecce, M.F., Censini, S., Covacci, A., Xiang, Z., 1994. Gene structure of the
638 Helicobacter pylori cytotoxin and evidence of its key role in gastric disease. J Exp
639 Med 179, 1653–1658. <https://doi.org/10.1084/jem.179.5.1653>

640 van Asseldonk, M., Rutten, G., Oteman, M., Siezen, R.J., de Vos, W.M., Simons, G.,
641 1990. Cloning of usp45, a gene encoding a secreted protein from Lactococcus
642 lactis subsp. lactis MG1363. Gene 95, 155–160. [https://doi.org/10.1016/0378-](https://doi.org/10.1016/0378-1119(90)90428-T)
643 1119(90)90428-T

644 Vandenbroucke, K., de Haard, H., Beirnaert, E., Dreier, T., Lauwereys, M., Huyck, L.,
645 Van Huysse, J., Demetter, P., Steidler, L., Remaut, E., Cuvelier, C., Rottiers, P.,
646 2010. Orally administered L. lactis secreting an anti-TNF Nanobody demonstrate
647 efficacy in chronic colitis. Mucosal Immunology 3, 49–56.
648 <https://doi.org/10.1038/mi.2009.116>

649 Vangay, P., Ward, T., Gerber, J.S., Knights, D., 2015. Antibiotics, Pediatric Dysbiosis,
650 and Disease. Cell Host Microbe 17, 553–564.
651 <https://doi.org/10.1016/j.chom.2015.04.006>

652 Vaucher, R. de A., Velho Gewehr, C. de C.V., Correa, A.P.F., Sant’Anna, V., Ferreira, J.,
653 Brandelli, A., 2011. Evaluation of the immunogenicity and in vivo toxicity of the
654 antimicrobial peptide P34. Int J Pharm 421, 94–98.
655 <https://doi.org/10.1016/j.ijpharm.2011.09.020>

656 Volzing, K., Borrero, J., Sadowsky, M.J., Kaznessis, Y.N., 2013. Antimicrobial Peptides
657 Targeting Gram-negative Pathogens, Produced and Delivered by Lactic Acid
658 Bacteria. ACS Synth. Biol. 2, 643–650. <https://doi.org/10.1021/sb4000367>

659 Voss, B.J., Gaddy, J.A., McDonald, W.H., Cover, T.L., 2014. Analysis of Surface-
660 Exposed Outer Membrane Proteins in Helicobacter pylori. J Bacteriol 196, 2455–
661 2471. <https://doi.org/10.1128/JB.01768-14>

662 Waterfield, N.R., Le Page, R.W., Wilson, P.W., Wells, J.M., 1995. The isolation of
663 lactococcal promoters and their use in investigating bacterial luciferase synthesis
664 in Lactococcus lactis. Gene 165, 9–15. [https://doi.org/10.1016/0378-](https://doi.org/10.1016/0378-1119(95)00484-n)
665 1119(95)00484-n

666 Wroblewski, L.E., Peek, R.M., Wilson, K.T., 2010. Helicobacter pylori and Gastric
667 Cancer: Factors That Modulate Disease Risk. Clin Microbiol Rev 23, 713–739.
668 <https://doi.org/10.1128/CMR.00011-10>

669 Xiong, Y.Q., Hady, W.A., Deslandes, A., Rey, A., Fraisse, L., Kristensen, H.-H.,
670 Yeaman, M.R., Bayer, A.S., 2011. Efficacy of NZ2114, a Novel Plectasin-
671 Derived Cationic Antimicrobial Peptide Antibiotic, in Experimental Endocarditis
672 Due to Methicillin-Resistant *Staphylococcus aureus* ▽. *Antimicrob Agents*
673 *Chemother* 55, 5325–5330. <https://doi.org/10.1128/AAC.00453-11>

674 Zarrinpar, A., Chaix, A., Xu, Z.Z., Chang, M.W., Marotz, C.A., Saghatelian, A., Knight,
675 R., Panda, S., 2018. Antibiotic-induced microbiome depletion alters metabolic
676 homeostasis by affecting gut signaling and colonic metabolism. *Nature*
677 *Communications* 9, 2872. <https://doi.org/10.1038/s41467-018-05336-9>

678 Zha, J., Liu, X.-M., Zhu, J., Liu, S.-Y., Lu, S., Xu, P.-X., Yu, X.-L., Liu, R.-T., 2016. A
679 scFv antibody targeting common oligomeric epitope has potential for treating
680 several amyloidoses. *Sci Rep* 6, 36631. <https://doi.org/10.1038/srep36631>

681 Zhang, L., Wu, W.K.K., Gallo, R.L., Fang, E.F., Hu, W., Ling, T.K.W., Shen, J., Chan,
682 R.L.Y., Lu, L., Luo, X.M., Li, M.X., Chan, K.M., Yu, J., Wong, V.W.S., Ng,
683 S.C., Wong, S.H., Chan, F.K.L., Sung, J.J.Y., Chan, M.T.V., Cho, C.H., 2016.
684 Critical Role of Antimicrobial Peptide Cathelicidin for Controlling *Helicobacter*
685 *pylori* Survival and Infection. *J Immunol* 196, 1799–1809.
686 <https://doi.org/10.4049/jimmunol.1500021>

687 Zhang, L., Yu, J., Wong, C.C.M., Ling, T.K.W., Li, Z.J., Chan, K.M., Ren, S.X., Shen, J.,
688 Chan, R.L.Y., Lee, C.C., Li, M.S.M., Cheng, A.S.L., To, K.F., Gallo, R.L., Sung,
689 J.J.Y., Wu, W.K.K., Cho, C.H., 2013. Cathelicidin protects against *Helicobacter*
690 *pylori* colonization and the associated gastritis in mice. *Gene Therapy* 20, 751–
691 760. <https://doi.org/10.1038/gt.2012.92>

692 Zorko, M., Jerala, R., 2010. Production of recombinant antimicrobial peptides in bacteria.
693 *Methods Mol Biol* 618, 61–76. https://doi.org/10.1007/978-1-60761-594-1_5

694