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Faculty of Natural Resources and Agricultural Sciences

Development of new food products with components active against Helicobacter pylori – with purpose to improve gastric health in humans

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Utveckling av nya livsmedelsprodukter med aktiva komponenter mot Helicobacter pylori i syfte att förbättra maghälsan hos människor

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PREFACE

Initiators of the project

The initiators of this project are the Associate Professor Halina Miller-Podraza, University of Gothenburg; Department of medical chemistry and cell biology, Food & Health Concept Centre (FHCC) and Skånemejerier. The project is based on the work of Halina Miller-Podraza and her discovery of the plant product PYLOS, active against *Helicobacter pylori*.

Treatment of sensitive information

The physiological parts of the compounds in the substrate PYLOS will not be discussed, nor will the recipes of the used food products from Skånemejerier. The products analyzed will be entitled with letters.

Abbreviations

PYLOS is the brand name of a plant substrate active against *H. pylori*. In this thesis the word PYLOS is used for resuming the two components; rosehip and walnut.

N = Rosehip
V = Walnut
FV = Freeze dried walnut extract

ABSTRACT

Gastric and intestinal disorders are common and costly human health problems worldwide. *Helicobacter pylori* are a gram-negative, pathogen bacteria and the most common cause of duodenal and gastric ulcer in the stomach the intestinal mucosa. Prolonged infection and colonization can lead to chronic gastritis and stomach cancer. *H. pylori* are present in the gastric and intestinal mucosa of half of the world population and a relation between infection and low socioeconomic status has been shown. Today the bacterial infection is treated with antibiotics and a proton pump inhibitor (e.g. Losec®). The interest in finding an alternative way of treating these bacteria is increasing, due to the risk of developing antibiotic resistance and high costs for medical drug treatments. Within Swedish research, a natural plant substrate, PYLOS, has been developed, which in laboratory tests and a clinical study, has been observed to inhibit adhesion and growth of *H. pylori* in the gastric and intestinal mucosa. The intention is to add the components of PYLOS to an existing food product in order to produce a *functional food* product effective against *H. pylori* infection.

This master thesis is based on a collaboration project between four Swedish actors; University of Gothenburg, Food & Health Concept Centre (FHCC), Skånemejerier and the Swedish University of Agriculture Science. The purpose has been to produce a product originating from Skånemejerier, but with addition of raw material and extracts of the plant substrate PYLOS. Two types of food products were used; juices and fermented milks. The activity in the product against *H. pylori* has been verified with microbiological analyses as hemagglutination-inhibition, where anti-adhesion of bacterial lectins to sialic acid-glycosylated conjugates on the cell surface was tested, as well as analyses of inhibited bacterial growth. The effect of the plant substrate was also tested after product heat treatment.

The results showed that PYLOS effectively inhibits *H. pylori* in juice products containing different fruit mixtures. Juice E, with its unique fruit composition, turned out to be the most effective product. Juice E (250 ml) totally inhibited the bacterial adhesion to erythrocytes diluted in a volume of 16-32 L and partially (about 50%) diluted in a volume of 32-256 L, depending on the bacterial strain tested. The analysis of bacterial anti-growth gave confirming results. However, PYLOS had lower inhibition capacity when added to fermented milk products. It is known that milk contains free sialylated oligosaccharides and sialylated glycoproteins. Theoretically, these can interfere with hemagglutination-inhibition tests. It is possible that sialylated milk compounds competed with sialylated saccharides present on erythrocytes resulting in a weaker inhibition of hemagglutination.

SAMMANFATTNING

Magsår och tarmbesvär hos människa är vanliga och kostsamma hälsoproblem världen över. *Helicobacter pylori* är en gramnegativ, patogen bakterie och den vanligaste orsaken till magsår och katarr i mag- och tarmslemhinnan. Långvarig infektion och kolonisering kan leda till kronisk magsjukdom och magcancer. *H. pylori* finns närvarande i mag- och tarmslemhinnan hos halva jordens befolkning och ett samband mellan infektion och låg socioekonomisk status har påvisats. Idag behandlas denna bakterieinfektion med antibiotika och en protonpumpshämmare (t.ex. Losec®). P.g.a. risker för antibiotisk resistensutveckling och höga läkemedelskostnader är intresset stort att finna ett alternativt sätt att behandla bakterieinfektionen. Inom svensk forskning har ett naturligt växtextrakt, PYLOS tagits fram, som i laborativa analyser och i klinisk studie visat sig hämma adhesion och tillväxt av *H. pylori* i mag- och tarmslemhinnan. Målet med PYLOS är att integrera substratet i ett existerande livsmedel och därigenom skapa ett mervärde, ett livsmedel med egenskaper aktiva mot *H. pylori*.

Detta examensarbete bygger på ett samarbetsprojekt mellan fyra svenska aktörer; Göteborgs Universitet, Food & Health Concept Centre (FHCC), Skånemejerier och Sveriges lantbruksuniversitet. Syftet har varit att genom produktutveckling ta fram en produkt med ursprung från Skånemejeriers sortiment som är lämplig för tillsats av växtextraktet PYLOS. Aktiviteten mot *H. pylori* hos de framtagna produkterna verifierades genom mikrobiologiska analyser som inhiberad tillväxt samt hemagglutination; där bakteriens anti-adhesion till sialinsyra-glykosylerade konjugat på cellens yta testades.

Två typer av livsmedel användes för detta ändamål; juicer och fermenterade mjölkprodukter. Råvaran till PYLOS och dess extrakt jämfördes, liksom PYLOS bevarade aktivitet efter att produkterna genomgått en värmebehandling.

Resultat visade att PYLOS effektivt inhiberar *H. pylori* i olika juiceprodukter och bäst resultat visade juicer innehållande kombinationen av två specifika frukter. Juice E om 250 ml innehållande en maximal koncentration av PYLOS, hämmade till 100% adhesionen av bakterien utspädd till en volym om 16-32 L. Adhesionen hämmades partiellt (ned till 50%) av produkten utspädd till en volym om 32-256 L. Tester av *H. pylori* anti-tillväxt gav överensstämmande resultat. PYLOS uppvisade sämre hämningsförmåga tillsatt i fermenterade mjölkprodukter. Det är känt att mjölk innehåller sialylerade oligosackarider och sialylerade glykoproteiner, vilka teoretiskt skulle kunna störa hemagglutinationshämmande tester. Det är möjligt att sialylerade mjölkföreningar konkurrerat med sialylerade sackarider som finns på erytrocyter, vilket vid analysförfarandet lett till svagare hämning av hemagglutination.

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1. INTRODUCTION

1.1. *Helicobacter pylori*

1.1.1. The bacterial characteristics and colonization in humans

Former theories stated that gastrointestinal diseases and ulcer was a result of stress and psychological unhealthy lifestyle. Twenty years ago, revolutionizing research reported that the symptoms caused by chronic gastritis, instead could be occasions of a bacterial infection. The discovery rendered the Nobel Prize in medicine in 2005 and since then, the scientific knowledge about *Helicobacter pylori* has grown remarkably (Arnqvist & Borén, 2003).

H. pylori cause one of the most common bacterial infections in the world. Half of the world population is thought to be carrier of the bacteria and about 33% of the Swedish population (Arnqvist & Borén, 2003). *H. pylori* is a 2-4 µl long, microaerophilic, spiral, flagellated and urease positive bacteria (Lin, *et al.*, 2005). It is physiological designed for its mobility and its ability to colonization in the mucus environment.

1.1.2. The gastro duodenal disease

An established infection causes inflammation in the gastric mucosa, called gastric catarrh. Some patients develop gastric ulcers in both the stomach and the duodenum (Arnqvist & Borén, 2003). Most of the carriers of the *H. pylori* bacteria have a mild chronic and asymptomatic inflammation, which does not require medical treatment. But 10-20% of the *H. pylori* infected individuals develop gastric diseases, with digestive syndromes like chronic inflammations and duodenal and gastric ulcer. Over 90% of all ulcer patients have infections caused by *H. pylori*. An infection is often life long without treatment and the probability to get symptoms increases with time exposed to the pathogenic cells.

H. pylori colonize naturally as a result of human contact with microbes. A relationship between socioeconomic standard and bacterial infection has been proven. Today the bacteria are usually common in developing countries, while a marked decline has been noticed in the industrialized part of the world (Marshall, 2002). *H. pylori* gastric colonization is known to start early in life. The mother or other family members are the most common sources of infection of small children, living in unsanitary conditions, without running water (Marshall, 2002). The bacteria spreads by the fecal-oral rout and possibly also the oral-oral route and by contaminated areas. Lower infection rate in industrialized countries are thought to be a result of improved sanity conditions, housing with fewer individuals and the increased use of antibiotics during childhood (Cover & Blaser, 2009). *H. pylori* have been classified by WHO (World health organization) as type 1-carcinogenic and are proved to cause stomach cancer in about 1-3% of the *H. pylori* positive patients (Miller-Podraza, *et al.*, 1997). In developing countries, gastric cancer is one of the most common forms of cancer (Marshall, 2002).

1.1.3. Biological factors that promote coexistence

There are several factors that enable existents of the bacteria in the stomach. The most important may be the *H. pylori* production of the enzyme ureas which can degrade the substance of urea. During such degradation, carbon dioxide and the basic compound ammonia are produced (Arnqvist & Borén, 2003). The ammonia is transformed to ammonium in presents of water and the remaining hydroxyl ion reacts with carbon dioxide, making bicarbonate which neutralizes gastric acid and makes the stomach environment survivable (Arnqvist & Borén, 2003).

Factors that also contributes to the coexistence is the location of the *H. pylori* in the mucus layer without substantial invasions of host tissue and its production of components that are adapted to reduce the human host immune response. One of those compounds is the special lipopolysaccharide (LPS), which with modifications of one lipid residue (cholesterol) makes the inflammation less intense. The bacteria is also capable of visualize itself as the adhesion molecules on the host cell to avoid the immune system to react, similar to the expression of the LPO 0 antigens that are structurally related to Lewis blood group antigen found in human cells. After infected the area, *H. pylori* are usually the dominant microorganism in the gastric region. It does have mechanisms to obtain valuable nutrients and produces peptides that reduce the competition from other microbes in the stomach. (Cover & Blaser, 2009)

1.1.4. The attachment to the cell

There are strain variations in how *H. pylori* are attached to the gastric epithelial cell surface. *H. pylori* have outer membrane proteins on its surface that function as adhesins; binding to membrane-associated lipids and carbohydrates. Several carbohydrate epitopes have been mentioned in the description of the adhesion of *H. pylori* to the cell surface receptors. These are called *BabA*, which binds to the fucoslylated Lewis b receptor displayed on the surface of the gastric epithelial cells and *SabA*, which binds to sialyl Lewis X receptors containing structures of sialyl acid (Cover & Blaser, 2009). Selected sialylated structures (containing N-acetylneuraminic acid) is said to be some of the most important adhesion factors (Miller-Podraza, 2009). These adhesion proteins are covered by the lipopolysaccharide molecule 0 (LPS 0) which structure is the same as Lewis blood group antigen, which makes human belong to blood group 0 according to the AB0-system. These similar systems with a modified cholesterol molecule could make the body to recognize *H. pylori* LPS as a component of its own (Cover & Blaser, 2009). According to Arnqvist and Borén (2002), this circumstances promotes the theory that people with blood group 0 is at greater risk of encounter the infection of *H. pylori*, due to the similar appearance of the sugar structures, where *H. pylori* could affect the immune system not to respond on the foreign bacteria.

1.1.5. Potential beneficial properties

H. pylori have also been associated with some beneficial properties in the body. Presence of *H. pylori* has been related with increased protection of allergy- induced asthma. Allergy scientists from Universities of Zurich and Gothenburg have in animal studies stated that the

increased level of asthma in industrial societies could be related to enhance of hygienically secured environments and the decreased infection rate of *H. pylori*. The bacteria have also been reported to counteract acid reflux disease due to the production of ammonium and bicarbonate in the stomach. (Arnold, *et al.*, 2011)

1.1.6. Clinical treatments used today

The treatment of *H. pylori* infected patients is based on the medical drug Losec®, an inhibitor of the proton pump that decreases the production of acid in the stomach. Losec® is consumed in combination with two different antibiotics during one week (Arnqvist & Borén, 2003). The risk for re-infection is low; about 0.5-2% of the treated patients gets re-infected per year. The practical issue of giving antibiotics to all infected patients is impossible, partly because of the heavy hypothetical cost, but also due to the increased risk of bacterial resistance. The use of antibiotics has resulted in a decreasing trend of peptic ulcer diseases, but it has also resulted in increased *H. pylori* resistance to some of the most used antibiotics (Cover & Blaser, 2009). Escalating resistance decreases the efficacy of the medical treatment and the pharmaceutical industry now calls for new alternative methods to treat the bacteria.

1.2. PYLOS

1.2.1. The project

The PYLOS-project started in 2006 as collaboration between the Associate Professor Halina Miller-Podraza at the Department of medical chemistry and cell biology at University of Gothenburg, Food & Health Concept Centre (FHCC) and Skånemejerier.

PYLOS is a natural plant substrate inhibiting *H. pylori* and preventing peptic ulcer diseases and in the extension, the development of digestive cancer. PYLOS has anti-adhesion properties based on the inhibition of the carbohydrate binding proteins, lectins, of *H. pylori* which bind to the glycoconjugates on the cell surface. Its anti-adhesion agents are chemically related to the epitopes on the target cells and restrain the binding of *H. pylori* (Miller-Podraza, 2009). PYLOS does also inhibit the bacterial growth and decreases the risk of inflammatory proliferation. The purpose of the product is to help people suffering from peptic ulcer to improve their digestive health, decrease the widespread use of antibiotics and contribute to a positive development of public health using natural ingredients. The natural ingredients are available on the market today which enables the option that people without digestive problems also will be able to consume the product. The vision of PYLOS is to be a respectful and leading actor within the field of inhibiting *H. pylori* (Food & Health Concept Centre, 2011).

1.2.2. The active components

Studies have shown that several plants contain a wide range of active compounds with ability to inhibit both growth and adhesion of *H. pylori*. PYLOS is the brand name of a specific mixture of rosehip and walnut powder/extract, scientifically proven to prevent the growth and attachment of *H. pylori* in the stomach lining (Miller-Podraza, 2009).

The compound/s exhibiting the inhibitory activity against *H. pylori* is thought to be polyphenoles, other antioxidants or proteins. Antimicrobial activities in key phenolic phytochemicals in plants have been detected in earlier studies (Lin, *et al*., 2005). Both rosehip and walnut shows activity against bacterial adhesion to sialic acid and growth of *H. pylori*, but both have special effects. Rosehip is known to mainly have anti-adhesion properties, one gram dried fruit has 100% inhibition of binding to sialic acid in the volume of 200 ml and partial inhibition in the volume of 400-800 ml. Walnut is better known for its inhibition of *H. pylori*-growth and has 100% inhibition in 40 ml and partial inhibition in 80-160 ml (Miller-Podraza, 2009). A number of other plants, fruits and berries have also been shown to have similar properties against the bacteria. An important aspect is their ability to complete and strengthen each other's inhibitory activities (Miller-Podraza, 2009).

Rosehip and walnut has earlier been investigated through treatment in different conditions. Those studies have shown that the activity of both plants are stable at low pH-environment (pH 1), but are sensitive for heat-treatment at higher temperatures (75°C for more than 30 s). Walnut is more sensitive than rosehip. (Miller-Podraza, 2011)

1.2.3. Clinical *in vivo* study

A pilot study on human was performed by Food & Health Concept Centre during year 2010-2011, where a combination of walnut and rosehip extract was given to *H. pylori* positive patients to confirm the same inhibiting effect *in vivo* as was proven *in vitro*.

In the study, 182 volunteers were tested for *H. pylori* infection. Only 14 persons showed a positive result in a test of antibody response through blood specimen collection and an urea breath test. The low infection frequency could be explained by the common use of antibiotics in our society. A treatment of antibiotics once in life means that a potential earlier *H. pylori* infection could have been cured. Eleven persons participated in the pilot study, where the hypothesis investigated was that PYLOS could prevent the bacterial attachment to the stomach lining and hinders further growth. Of these eleven persons, seven were females and four were men. None of the patients had earlier awareness of their *H. pylori* infection and none have had symptoms related to it. The patients were given 8 g rosehip and 8 g walnut powder twice-a-day during 30 days. After 30 days of treatment, the effect was shown in an exhalation test, where urea was measured. Of the eleven persons treated, 73% (8 of 11) had on average halved their amount of *H. pylori*. Two persons (18%) were shown to be free from the *H. pylori*-infection and one person did not respond to the treatment. (Björkman, 2011)

1.2.4. Functional foods

PYLOS as food ingredients and dietary supplements are planned for health concept food stores and pharmacies. The consumers of today are anxious of their health and the demand of products with added values increases. Functional foods are food with scientifically proven active ingredients that has beneficial physiological effects in the body, beyond the ordinary nutritional values (Blücher, 2003). The product could be an innovation, but is often an improvement of an already existing product on the market, with valuable additives and a health-promotion. Functional foods are considered as product on the boundary between foods

and medicine. The foods are a part of a strong global health trend with strong drive in USA and Europe. Qualified functional foods is a good alternative for the society and the individual to prevent diseases that originates in the industrialized part of world, like high blood pressure, problems with digestive health, diabetes and obesity (Bruce, 2000). A good market potential can be expected for functional food products. The market is growing with about 10-20% per year with a continued increasing demand (Food & Health Concept Centre, 2011).

2. AIM

The purpose with this master thesis has been to develop a product, originating from the range of Skånemejerier but with addition of the plant substrate PYLOS*,* and evaluate the activity against *H. pylori* using verifying microbiological analyses.

The study is based on following question formulation;
How is the activity of PYLOS against H. pylori affected after addition to different liquid food products?

3. MATERIAL & METHODS

3.1. Study design

The study was divided in two parts. The first part involved product development, where PYLOS was added to already existing products of Skånemejerier and taste, consistency, color etc. were evaluated in a descriptive sensory test. Small batches of 16 products with four different concentrations of PYLOS were produced. In the second part, the inhibition activity of the new products were tested against *H. pylori* with verifying microbiological analyze methods at University of Gothenburg.

3.2. Materials

3.2.1. The PYLOS substrate

Particular requirements were requested for the raw material; rosehip (*Rosa canina*) and walnut (*Juglans regia*), which were carefully selected from specific suppliers. Walnut powder contains large particles with all natural components included. The walnut powder has also been compared with freeze dried walnut powder extract. The extract, is walnut powder extracted with water and freeze dried. This report will in the following mention the use of PYLOS substrate as rosehip and walnut (N+V) in weight relationship 1:1 respectively rosehip and freeze dried walnut extract (N+FV) in weight relationship 1:0.2. The two combinations were used in different products. FV had a higher activity/g than V and was therefore used in lower amount, 1 g V = 0.2 g FV (Miller-Podraza, 2011).

3.2.2. Juice concentrates

The juice products A, B, C, D, E and F were all specific beverages containing fruit concentrates with different components. The juices were natural products, containing 100% fruits and no added sugar, produced at the dairy of Skånemejerier.

3.2.3. Fermented milk products

The products G, H, I and J were fermented milks with 0.5-1% fat. The products were fermented using different starter cultures which resulted in unique characters in terms of taste and texture. The fermented milk products were all chosen with low amount of fat, to avoid too thick texture when adding PYLOS.

3.3. Experiments and analyses

3.3.1. Product development

The product development procedure was done as a small-scale sensory test in the lab of product development at Skånemejerier, Lunnarp. Small batches of 250 ml were produced through mixing fruit concentrate with water to right concentrations, following the guideline of Skånemejerier, and adding substrate of PYLOS in four different concentrations. Fermented milk products with PYLOS added, were also developed.

According to the clinical *in vivo* study, 8 g rosehip and 8 g walnut twice-a-day had positive effects against *H. pylori* in infected patients (Björkman, 2011). 16 g rosehip and 16 g walnut was therefore used as the maximal dose of PYLOS substrate in the products produced. To analyze the optimal activity level of PYLOS, the concentration of substrate was also added as divided to the half (8 g + 8 g), a quarter (4 g + 4 g) and an eighth parts (2 g + 2 g) of the maximal dose for every product (Table 1).

Addition of PYLOS substrate was done in both juices and the fermented milk products. In the juice samples, walnut was added both as powder (V) and as freeze dried walnut extract (FV). However, the FV was difficult to solve and therefore the juice product C was warmed to 48°C in order to increase the solubility.

Sensory tests

The sensory test was performed as a descriptive Free Choice Profiling test. In a Free Choice Profiling test, each assessor creates his/her own attributes for the product and no panel training needs to be done (Lawless & Heymann, 2010). The assessors were allowed to evaluate the product in different ways and the data was then summarized. The assessors used were an internal panel of three people at the product development unit of Skånemejerier. The panel was presented samples of products with four different concentrations of PYLOS, asked to choose in which concentration of PYLOS the product was acceptable. Acceptable meant in this study that the product tasted good, looked good or represented any other positive opinion

including greater satisfaction. The panel was also asked to answer which flavors that were prominent and less prominent in the different concentrations and in which degree the products tasted bitter on a 1-4 degree scale. The tested samples are shown in Table 1. These samples were then analyzed for activity against *H. pylori* in the verifying microbiological tests.

Table 1 Produced samples of juices and fermented products with PYLOS substrate added

Products	PYLOS substrate	PYOS (g) /250ml	PYLOS (g) /250ml	PYLOS (g) /250ml	PYLOS (g) /250ml
Juice A-F	N+V	16+16	8+8	4+4	2+2
Juice A-E	N+FV	16+3.2	8+1.6	4+0.8	2+0.4
Fermented milk G-J	N+V	16+16	8+8	4+4	2+2

3.3.2. Verifying microbiological analyses

The microbiological analyses were divided in two analytical methods to study different inhibition mechanisms of PYLOS.
1.) A hemagglutination test was performed to investigate the inhibition effect of the PYLOS product against the adhesion of *H. pylori* to human erythrocytes through interactions with membrane bound sialylated carbohydrate structures. Inhibition of agglutination was evaluated using light microscopy (Miller-Podraza, *et al.*, 1997).
2). A growth inhibition test on agar plates was performed to detect anti-growth properties of the PYLOS products.

Before analysis the products was filtrated, in purpose to eliminate lumps and to get a thinner consistency. The product was warmed at room temperature and shaken. The sample was filtered with filter paper grade 113 µm (Whatman, Buckinghamshire, UK) under vacuum pressure.

1). Hemagglutination inhibition assay

The hemagglutination inhibition tests were started by separating the human erythrocytes from a fresh human blood sample (blood type 0) through washing with Phosphate-Buffered Saline (PBS). The sample was centrifuged 3 times at 1.5 rpm during 6 min discarding the supernatant.

Culture tissue plates with 96 wells (Sarstedt AB, Germany) were used in the analyses (follow the method in Table 2). The first row, A1-A12, was used as *H. pylori*-controls wells where A1-A6 was negative controls (*H. pylori* not included) and A7-A12 were positive controls (*H. pylori* included). 38 µl PBS was added in the negative control and 25 µl PBS was added in the positive control. The wells B1-H12 were filled with 25 µl PBS. In well C1-H1 the filtrated product containing PYLOS was added in a volume of 50 µl and mixed with the PBS. The products were compared with a PYLOS-control (B1-B12), wells with 50 µl of N+V or N+FV due to the content of the investigated product. The products and the PYLOS-control were then diluted two-folded by transferring 25 µl from B1-H1 to B2-H2 and mixing. Then

25 µl was taken from B2-H2 and transferred to B3-H3 and was mixed, etc. until a dilution of 2058 was reached in every row (Table 2). The plate was incubated for 0.5-1 hour at room temperature. After incubation, 50 µl of the erythrocytes was diluted with 5 ml PBS to a concentration of 1% and 25 µl of the solution was added to all wells. One colony of *H. pylori* was diluted in 11 ml PBS and 12.5 µl of the solution was added in all wells but the negative control. The plate was incubated at room temperature for 1.5-2 hours. The same procedure was done with all the products.

Table 2 The table illustrates the culture tissue plate in which the hemagglutination assay was performed

2-fold dilution												
Dilution	0	2	4	8	16	32	64	128	256	512	1024	2058
Well	1	2	3	4	5	6	7	8	9	10	11	12
A	C.[1] Ery.[2]	PBS + Ery[2]					PBS + Ery[2] + *H. pylori*					
B	C.[1] PYLOS											
C	Product 1											
D	Product 1											
E	Product 2											
F	Product 2											
G	Product 3											
H	Product 3											

[1] C. = Control, [2]Ery. = Erythrocytes

Every product was analyzed twice. Two different strains of *H. pylori* were used in every test to increase the reliability of the results. *H. pylori* CCUG 17874 was used in all experiment as a standard strain. In addition another strain, J99, was used to verify the result. Both strains came from the culture collection of University of Gothenburg.

The reaction was observed through examination a small volume from every well in the microscope. When the bacteria attached to the cell, agglutination occurred that was seen as big aggregated flaks of erythrocytes. When the product including PYLOS was added, the agglutination was inhibited and the bacteria did not attach to the cell. Only free erythrocytes observed in the microscope indicated complete inhibition of agglutination; the inhibiting product was said to work anti-adhesive. No free erythrocytes observed, indicated total agglutination; the inhibiting product was diluted to a rate where it was no longer effective.

Table 3 shows an example of a reaction evaluation of a culture tissue plate using the microscope. The rate of agglutination was subjectively estimated and graded at a 4-degree scale illustrated with pluses, where "-" meant no agglutination (100% inhibition) and "++++" meant full agglutination (0% inhibition). About 50% inhibition was illustrated with "++". This method showed in which volume the products with PYLOS inhibited *H. pylori* adhesion with 100%, 50% and 0%.

Table 3 The table shows an example of an evaluation of a culture tissue plate when grading the rate of agglutination in the microscope

2-fold dilution												
Dilution	0	2	4	8	16	32	64	128	256	512	1024	2058
Well	1	2	3	4	5	6	7	8	9	10	11	12
A	C.[1] Ery.[2]	-	-	-	-	-	++++	++++	++++	++++	++++	++++
B	C.[1] PYLOS	-	-	-	-	-/+	+	++	+++	++++	++++	++++
C	Prod[3] max[4]	-	-	-	+	++	+++	++++	++++	++++	++++	++++
D	Prod[3] max[4]	-	-	-	+	++	+++	++++	++++	++++	++++	++++
E	Prod[3] med[5]	-	-	+	++	+++	++++	++++	++++	++++	++++	++++
F	Prod[3] med[5]	-	-	-/+	++	+++	++++	++++	++++	++++	++++	++++
G	Prod[3] min[6]	-	-/+	++	+++	++++	++++	++++	++++	++++	++++	++++
H	Prod[3] min[6]	-	+	++	+++	++++	++++	++++	++++	++++	++++	++++

[1] C. = Control, [2]Ery. = Erythrocytes, [3]Prod. = Product, [4]Max = Maximal concentration, [5]Med = Medium concentration, [6]Min = Minimal concentration, "-" = No agglutination (100% inhibition), "+" = Weak agglutination, "++" = Partial agglutination (50% inhibition), "+++" = Strong agglutination, "++++" = Total agglutination, (no inhibition)

2). Growth assay

The growth inhibition tests of *H. pylori* were done using 3 ml agar-gel on agar plates, 3.5 cm in diameter (Sarstedt AB). The inhibitory product was filtrated through sterile filters (filtropur S-plus, 0.2 µm, Sarstedt AB), to avoid bacterial growth which could compete with *H. pylori*. The PYLOS product was diluted to 1:1 and 1:2 and applied in a volume of 200 µl on the surface of the agar plates and the product was allowed to sink into the gel overnight. A plate applied with water (200 µl) made the control sample for the bacterial growth and an addition of N+V/N+FV (200 µl) was the inhibition control. The *H. pylori* cells were streaked on the agar plate and the bacteria were allowed to grow for two days at 37°C. If inhibition appeared after two days, further dilutions of the product were tested (i.e. 1:4 and 1:8) to determine at which dilution the inhibition effect disappeared.

Table 4 shows an example of how the agar plates were analyzed after incubation. The results were read in the same way as in the hemagglutination assay. The rate of growth was subjectively estimated and graded at a 4-degree scale illustrated with pluses, where "-" meant no growth (100% inhibition) and "++++" meant full growth (0% inhibition). About 50% inhibition was illustrated with "++". This method showed in which volume the products with PYLOS inhibited *H. Pylori* growth with 100%, 50% and 0% efficiency. The growth assay was performed on a limited number of products, only products with maximal concentrations.

Table 4 The table shows an example of an evaluation of grading the rate of growth on agar-plates

2-fold dilution												
Dilution	0	2	4	8	16	32	64	128	256	512	1024	2058
Well	1	2	3	4	5	6	7	8	9	10	11	12
A	C.[1] Ery.[2]	-	-	-	-	-	++++	++++	++++	++++	++++	++++
B	C.[1] PYLOS	-	-	-	-	-/+	+	++	+++	++++	++++	++++
C	Prod[3] max[4]	-	-	+	++	+++	++++	++++	++++	++++	++++	++++
D	Prod[3] max[4]	-	-	+	++	+++	++++	++++	++++	++++	++++	++++
E	Prod[3] med[5]	-	+	++	+++	++++	++++	++++	++++	++++	++++	++++
F	Prod[3] med[5]	-	-/+	++	+++	++++	++++	++++	++++	++++	++++	++++
G	Prod[3] min[6]	+	++	+++	++++	++++	++++	++++	++++	++++	++++	++++
H	Prod[3] min[6]	+	++	+++	++++	++++	++++	++++	++++	++++	++++	++++

[1] C. = Control, [2]Ery. = Erythrocytes, [3]Prod. = Product, [4]Max = Maximal concentration, [5]Med = Medium concentration, [6]Min = Minimal concentration, "-"= No growth (100% inhibition), "+" = Weak growth, "++" = Partial growth (50% inhibition), "+++" = Strong growth, "++++" = Total growth, (no inhibition)

Treatment of controls

The controls used were extracts of N+V with concentration ratio 1:1 and N+FV with concentration ratio 1:0.2 for products including N+V and N+FV, respectively. The controls were made by making dilutions in water of N, V and FV. The controls were shaken and kept overnight before filtrated. The same controls were used for all products and were kept in a freezer when not used. New bacterial colonies were used every day and since the quality and agglutination strength of *H. pylori* could vary, a control analyze was performed with every new bacterial colony used. The controls of PYLOS had a higher concentration than in the product and were therefore adjusted with a regulation factor to correspond to the concentration of PYLOS in the products (*Mathematical models*).

Investigative tests of fermented milk products

During the experimental period, an obvious decrease of the inhibition activity of PYLOS was observed in the fermented milk products. Several investigative tests were performed to study where in the process the activity was lost. Only *H. pylori* strain CCUG 17874 was used in these tests, which are summarized in Table 5.

To eliminate the option that the product had not been produced properly, new samples with PYLOS substrate added to product H were made. Different filtration methods were used to see if any technical factor was responsible for the loss of activity. The filter paper was changed; a large amount of substrate could have been lost during the filtrating process. The product was also extracted by dilution and centrifugation. Different concentrations of PYLOS were tested to determine if the amount of substrate was important for the preservation of activity. The incubation times were also varied, during which the mixture was shaken before filtration. Samples were also mixed with erythrocytes to exclude the option that the milk itself may agglutinate the erythrocytes. In one test hydrochloric acid (HCl) was added to a sample to denature the milk proteins further. The sample was centrifuged and the proteins were discarded before the sample was neutralized with sodium hydroxide (NaOH) and analyzed with hemagglutination. Sodium chloride (NaCl) was also added to a sample before analyze to eliminate the option that osmosis affected the erythrocytes in the previous test

($HCl + NaOH \rightarrow NaCl + H_2O$). All tests were performed using product H with maximal concentration of PYLOS. The control used in these tests was the origin product H with maximal concentration of PYLOS.

Table 5 Following tests were done to investigate the loss of activity in the fermented milk products

Test	Method
New mixture	PYLOS was mixed with product H once more to make sure that the mixture was done properly.
Filter paper	Filter paper 113 µm (Whatman) was used in the filtration procedure.
Filter paper	Filter paper (thin) (Whatman) was used in the filtration procedure
Filter paper	Filter paper (thick) (Whatman) was used in the filtration procedure.
Centrifugation	Centrifugation was used instead of filtration (3 x 4.0 rpm)
Dilution	The product was diluted 2 times and incubated during 4 h, before filtration with a thin filter paper.
Dilution	The product was diluted 2 times and incubated over night before filtration with a thin filter paper.
No filtration	The product was not filtrated at all before analyze.
Change of conc[7]	32 g PYLOS (1:1) was mixed with 250 ml product and incubated during 5 h before analyze.
Change of conc[7]	16 g PYLOS (1:1) was mixed with 250 ml product and 16 ml PBS and incubated during 5 h before analyze.
Change of conc[7]	8 g PYLOS (1:1) was mixed with 250 ml product and 25 ml PBS and incubated during 5 h before analyze.
Change of inc[9] time	32 g PYLOS (1:1) was mixed with 250 ml product. 10 ml was incubated during 0.5 h before analyze.
Change of inc[9] time	10 ml of the product above was incubated during 4 h before analyze.
Change of inc[9] time	10 ml of the product above was incubated over night before analyze.
Sample + Ery[2]	Erythrocytes were added to the product without *H. pylori*.
HCl added	3 M HCl was added to the product filtrate to reach pH 1. The product was centrifuged and proteins removed. 3 M NaOH was added to reach pH 4.5.
NaCl + Ery[2] + Hp[8]	3 M NaCl was added to erythrocytes and *H. pylori*.

[2]Ery = Erythrocytes, [7]Conc. = Concentration, [8]Hp = *H. pylori*, [9]Inc. = Incubation

Heat treatment

When producing fermented milk, the milk is heat treated at 95°C for about 10 min before addition of lactic acid bacteria. Juice products are also pasteurized at 90°C for 10 s before packaging. When adding PYLOS to a food product, it has to be sure that even the PYLOS substrate is free from pathogenic bacteria and therefore it would be an advantage if PYLOS could be added to the product before the heat treatment. A heat treatment test was thus done to find out how the inhibition of *H. pylori* was affected by heating PYLOS.

Heat treatment was done in small scale using a waterbath on an induction cooker. The induction regulation made it easy to adjust the temperature, which was controlled by a thermometer. The juice C with PYLOS substrate N+V (16 g + 16 g) and N+FV (16 g + 3.2 g) and milk (0.5% fat) with PYLOS substrate N+V (16 g + 16 g) and N+FV (16 g + 3.2 g), were heated in eppendorf-tubes in waterbath. The temperature and time used was adapted to standard heat treatment procedures at the dairy; i.e. low pasteurization (72°C for 15 s), high pasteurization (85°C for 5 s) and heat treatment of milk which will be fermented (95°C for 10 min) (Walstra, *et al.*, 2006). The samples were placed on icebath after heating. The juices were pasteurized at 90°C for 10 s in accordance with the procedure of Skånemejerier. The control used in this test was the same product, not heat treated.

3.4. Mathematical models

3.4.1. Verifying microbiological analyses

The inhibition effect of PYLOS *by volume*

Since the target of the product is the stomach, it is important to know at which volume the product is inhibitive. Table 6 shows an example of an evaluation of a hemagglutination assay.

The concentrations of rosehip and walnut substrate have been considered as the concentration of PYLOS (N+V or N+FV), to simplify the calculations and to visualize that the two substrates are acting together against *H. pylori*.

The effect of PYLOS has been calculated as the following example:

A product with maximal concentration:

16 g (N) + 16 g (V) in 250 ml = 32 g PYLOS in 250 ml.

32 g / 250 ml = 1 g / 7.81 ml

100% inhibition ended in a dilution up to 4 times (Table 6).

4 x 7.81 = 31.25 ml → 1 g PYLOS was effective in 31.25 ml

32 g PYLOS used gives 31.25 x 32 = 1000 ml = 1 L

50% inhibition ended in a dilution up to 16 times.

16 x 7.81 = 125 ml → 1 g PYLOS is effective in 125 ml

32 g PYLOS used gives 125 x 32 = 4000 ml = 4 L

The product with maximal concentration has 100% inhibition against *H. pylori* adhesion in a volume up to 1 L and 50% inhibition in a volume up to 4 L.

Table 6 The table shows an example of an evaluation used when grading the rate of agglutination in the microscope

2-fold dilution										
Dilution	0	2	4	8	16	32	64	128	256	512
Well	1	2	3	4	5	6	7	8	9	10
A	C.[1] PYLOS	-	-	-	-	-	+/-	+	++	+++
B	Prod[3] max[4]	-	-	+	++	+++	++++	++++	++++	++++
C	Prod[3] med[5]	-	+	++	+++	++++	++++	++++	++++	++++
D	Prod[3] min[6]	+	++	+++	++++	++++	++++	++++	++++	++++

[1]C. = Control, [3]Prod. = Product, [4]Max = Maximal concentration, [5]Med = Medium concentration, [6]Min = Minimal concentration, "-"= No growth (100% inhibition), "+" = Weak growth, "++" = Partial growth (50% inhibition), "+++" = Strong growth, "++++" = Total growth, (no inhibition)

Treatment of controls

The controls had a higher concentration of PYLOS than the products. Therefore the activities of the controls had to be re-calculated to be comparable with the activities of the analyzed products. The dilution, in which the inhibition effect of the product decreased, was divided by the regulation factor. The regulation factor is the ratio between the concentration of PYLOS in the control and the concentration of PYLOS in the product.

The effect of the controls has been calculated as in the following example where PYLOS substrate N+V is used;

Rosehip extract (N = 0.1 g/ml) and walnut extract (V = 0.3 g/ml) were mixed to a concentration ratio of 1:1 with the total amount of 32 g (maximal concentration of PYLOS).

24.7 g (N) + 7.4 g (V) in 96 ml = 32 g PYLOS in 96 ml

32 g / 96 ml = 1 g in 3 ml

The control was 100% effective up to a dilution of 32 times. According to Table 7, the regulation factor of N+V at maximal concentration of PYLOS is 2.58.

32 / 2.58 = 12.4

12.4 x 3 = 37.2 ml → 1 g PYLOS was 100% effective in 37.2 ml

32 g used gives 37.2 x 32 = 1190 ml ~ 1.2 L

The control gave 50% inhibition up to a dilution of 256 times.

256 / 2.58 = 99.2

99.2 x 3 = 297.7 ml → 1 g PYLOS was 50% effective in 297.7 ml

32 g used gives 297.7 x 32 = 9526 ml ~ 9.5 L

The control has 100% inhibition against *H. pylori* adhesion in a volume up to 1.2 L and 50% inhibition up to 9.5 L.

Table 7 Products with PYLOS substrate N+V. The regulation factor is the ratio between concentration of control and concentration of product

g PYLOS/ ml product	Conc. of PYLOS in the product (g/ml)	Conc. of PYLOS in the control (g/ml)	Regulation factor
16+16/250	0.128	0.33	2.58
8+8/250	0.064	0.33	5.16
4+4/250	0.032	0.33	10.31
2+2/250	0.016	0.33	20.63

[7]Conc = Concentration

Table 8 Products with PYLOS substrate N+FV. The regulation factor is the ratio between concentration of control and concentration of product

g PYLOS/ ml product	Conc. of PYLOS in the product (g/ml)	Conc. of PYLOS in the control (g/ml)	Regulation factor
16+3.2/250	0.077	0.167	2.17
8+1.6/250	0.038	0.167	4.35
4+0.8/250	0.019	0.167	8.70
2+0.4/250	0.01	0.167	17.40

[7]Conc = Concentration

4. RESULTS

4.1. Product development

4.1.1. Juices N + V

The pH of these products was between 3.45 and 4. The addition of PYLOS substrate increased the pH of the product linearly. The juice products D and E had a lower pH where juice E had the lowest. The temperatures of the juices were measured to 17-23°C at the time of tasting.

In the products with maximal dose of N+V, the rosehip was difficult to dissolve. It floated on the surface, got clumpy and required a lot of mixing. The solubility of the powder increased with less powder added. The viscosity was high in the products with maximal concentration and decreased with lower concentration.

The walnut powder caused problems with the consistency of the product. Due to its high fat content, big particles float on the surface and gave an unattractive sedimentation layer. The big particles were also noticeable when drinking the juice. They gave an unpleasant mouth feel and tasted very bitter when chewed. The bitterness of the walnut was more palpable at higher concentrations and decreased with lower amount added. Some of the juices hid the bitter taste even at higher concentrations, i.e. juice B and E.

The colour of the products did also change with the concentration of PYLOS substrate. At maximal concentration the walnut had a strong influence of the colour and many of the juices turned in to brown. That colour was not attractive in juices, which were normally light orange coloured. Their appearances were also thick, brown and opaque. In juice E, the purple colour hid the discoloration of the walnut. The juices having the best ratings in this test were juice E and B. Table 9 shows the results of the descriptive sensory test of the juices containing PYLOS substrate N+V. Bitterness is graded in a 4-degree scale where 4 is most bitter. The general score is graded at a 4-degree scale where 4 are the highest score. Highest acceptable concentration is marked with an “x”.

Table 9 The table shows the results from the product development of juices containing PYLOS substrate N+V

Juice	Conc[7] (N+V)g	Colour	Prominent taste	Bitterness (1-4)	General Score (1-4)	
A	16+16	Orange/brown/yellow	Rosehip/walnut	3	1	
A	8+8	Yellow/Orange/brown	Walnut/M.c.[10]	3	2	
A	4+4	Yellow/Orange/ brown	M.c.[10]/Rosehip	1	3	
A	2+2	Yellow/Orange	M.c.[10]	1	4	x[12]
B	16+16	Orange/light brown	S.m.c.[11]/walnut	3	2	
B	8+8	Orange/light brown/yellow	S.m.c.[11]/rosehip	2	3	
B	4+4	Yellow/Orange/ brown	S.m.c.[11]	1	4	x[12]
B	2+2	Yellow/Orange	S.m.c.[11]	1	4	
C	16+16	Brown/Orange	Walnut/rosehip	4	1	
C	8+8	Light brown/Orange	Walnut/rosehip	4	2	
C	4+4	Orange/brown	M.c.[10]/rosehip	3	3	
C	2+2	Yellow/Orange	M.c.[10]/rosehip	2	3	x[12]
D	16+16	Dark brown	Walnut/rosehip	4	1	
D	8+8	Brown	M.c.[10]	3	2	
D	4+4	Light brown	M.c.[10]	3	3	
D	2+2	Light brown	M.c.[10]	2	3	x[12]
E	16+16	Dark purple	M.c.[10]/rosehip	2	2	
E	8+8	Purple	M.c.[10]	2	3	x[12]
E	4+4	Purple	M.c.[10]	1	4	
E	2+2	Purple	M.c.[10]	1	4	
F	16+16	Brown	Walnut/apple	4	1	
F	8+8	Light brown/yellow	M.c.[10]/walnut	3	2	
F	4+4	Yellow/grey/uncolored	M.c.[10]/S.m.c.[11]	1	3	x[12]
F	2+2	Yellow/grey/uncolored	M.c.[10]/S.m.c.[11]	1	4	

[7]Conc. = Concentration, [10]M.c. = Main component of the product, [11]S.m.c = Second main component of the product
[12]x = Highest acceptable concentration

4.1.2. Juices N + FV

The pH of these products was between 3.25 and 3.58. The addition of PYLOS substrate increased the pH of the product linearly as for the juices with N+V. The juices D and E had a low pH where juice E had the lowest. The temperature of the juices was 17-23°C at the time of tasting.

In products containing N+FV, the rosehip was difficult to dissolve, but in these products the freeze dried walnut was even harder to dissolve and were visualized as brown spots in the juice. The freeze dried walnut extract did also float on the surface, but was not visible in the

same extent as the walnut powder. The juice product C, with highest concentrations of PYLOS substrate was heated to 48°C for 10 s which facilitated the solubility and made the character smoother and planer. Using a homogenizer did also facilitate the solubility. No sedimentation was caused by the freeze dried walnut extract. The bitter taste was less significant using the walnut extract compared with the walnut powder and the bitterness decreased with the lower amount added or by warming the juice. It was also noticed that some product flavors hid the bitterness more than others. Adding the PYLOS substrate N+FV due to the low amount extract used did not discolour the juices. Table 10 shows the results of the descriptive sensory tests of the product development procedure of the juices containing PYLOS substrate N+FV.

Table 10 The table shows the results from the product development of juices containing Pylos substrate N+FV

Juice	Conc.[7] (N+FV)g	Colour	Prominent taste	Bitterness (1-4)	General score (1-4)	
A	16+3.2	Orange/brown/yellow	Rosehip/walnut	4	1	
A	8+1.6	Yellow/orange	Walnut/M.c.[10]	3	2	
A	4+0.8	Yellow/orange	M.c.[10]/rosehip	1	3	x[12]
A	2+0.4	Yellow/orange	M.c.[10]	1	4	
B	16+3.2	Orange/light brown	S.m.c.[11]/walnut	3	1	
B	8+1.6	Orange/yellow/brown	S.m.c.[11]/rosehip	2	2	
B	4+0.8	Orange/yellow	S.m.c.[11]	1	3	x[12]
B	2+0.4	Yellow/orange	S.m.c.[11]	1	4	
C	16+3.2	Brown/orange	Walnut/rosehip	4	2	
C (h)[13]	16+3.2	Brown/orange	M.c.[10]/walnut	2	2	
C	8+1.6	Light brown/orange	Walnut/rosehip	3	3	
C (h)[13]	8+1.6	Light brown/orange	Walnut/rosehip	1	3	x[12]
C	4+0.8	Yellow/orange	M.c.[10]/walnut	2	4	
C	2+0.4	Yellow/orange	M.c.[10]/rosehip	1	3	
D	16+3.2	Dark brown	Walnut/rosehip	4	1	
D	8+1.6	Brown	M.c.[10]	3	2	
D	4+0.8	Light brown	M.c.[10]	2	3	x[12]
D	2+0.4	Light brown	M.c.[10]	2	4	
E	16+3.2	Dark purple	M.c.[10]/rosehip	2	2	
E	8+1.6	Purple	M.c.[10]/S.m.c.[11]	1	3	x[12]
E	4+0.8	Purple	M.c.[10]/S.m.c.[11]	1	4	
E	2+0.4	Purple	M.c.[10]/S.m.c.[11]	1	4	

[7]Conc = Concentration, [10]M.c. = Main component of the product, [11]S.m.c. = Second main component of the product, [12]x = Highest acceptable concentration, [13](h) = Heated at 48°C for 10s

4.1.3. Fermented milk products N + V

The different fermented products had varying pH between 4.22-4.55 with product J at the lowest. The addition of PYLOS substrate increased the pH linearly as for the juice products. The temperature of the products was measured to 20-21°C at the time of tasting.

The products tested had natural taste and almost the same fat content, which made the results of the sensory tests similar. The rosehip powder was difficult to dissolve, but was not experienced as clumping due to the thicker consistency of the fermented product. The viscosity increased when adding the PYLOS substrate, but did not get too thick thanks to the low fat content. The consistencies were regarded as pleasant and were considered as best in higher concentrations. The products were comparable with a between meal-snack, i.e. PYLOS substrate was a good substitute for muesli. The particles did not give an unpleasant mouth feel, as the juices containing N+V. On the contrary, the particles increased the tasting experience of the product.

The colour of the products was only changed slightly orange with increasing concentration of PYLOS. However, the colour change was not a disadvantage for the appearances of the products. At lower concentrations, the PYLOS substrate was just visualized as orange and brown spots in the white product.

Flavours of both rosehip and walnut could be recognized in the products at higher concentrations, but the fermented milks hid all taste of bitterness. The addition of PYLOS did actually increase the overall impression of the product and regarding the general score (Table 11), the products with higher concentrations of PYLOS were generally better than those with lower concentration. The product J got the lowest general score in the sensory test, since the sour taste did not match the taste of walnut properly.

Table 11 The table shows results from the product development of fermented milk products containing PYLOS substrate N+V

Fermented milk	Conc. (N+V)g	Colour	Prominent taste	Bitterness (1-4)	General score (1-4)	
G	16+16	W[13]/orange/light brown	Walnut/rosehip	1	3	x
G	8+8	W[13]/orange/light brown	Rosehip/walnut	1	4	
G	4+4	W[13]/orange/brown spots	M.c.[10]	1	2	
G	2+2	W[13]/orange/brown spots	M.c.[10]	1	1	
H	16+16	W[13]/orange/light brown	Walnut/rosehip	1	3	x
H	8+8	W[13]/orange/light brown	Rosehip/walnut	1	4	
H	4+4	W[13]/orange/brown spots	M.c.[10]	1	2	
H	2+2	W[13]/orange/brown spots	M.c.[10]	1	1	
I	16+16	W[13]/orange/light brown	Walnut/rosehip	1	3	x
I	8+8	W[13]/orange/light brown	Rosehip/walnut	1	4	
I	4+4	W[13]/orange/brown spots	M.c.[10]	1	2	
I	2+2	W[13]/orange/brown spots	M.c.[10]	1	1	
J	16+16	W[13]/orange/light brown	M.c.[10]/walnut/rosehip	1	2	
J	8+8	W[13]/orange/light brown	M.c.[10]/walnut/rosehip	1	3	
J	4+4	W[13]/orange/brown spots	M.c.[10]	1	3	x
J	2+2	W[13]/orange/brown spots	M.c.[10]	1	3	

[10]M.c. = Main component of the product, x[12] = Highest acceptable concentration, W[13] = White

4.2. Verifying microbiological analyses

4.2.1. Hemagglutination inhibition assay

All products were analyzed with two different bacterial strains, *H. pylori* CCUG 17874 (74) and J99 (99). In the Tables 12-14, the volume in which the product inhibits the bacteria is shown as an interval of two different volumes. The first value corresponds to the effective volume for *H. pylori* 74 and the second volume corresponds to the effective volume for *H. pylori* J99. *H. pylori* 74 are know to have a stronger agglutination than J99 (Miller-Podraza, 2011). This means that the inhibition was less effective for *H. pylori* 74 and the inhibition volume was lower. In an inhibition volume between e.g. 4000-8000 ml, the bacterial strain 74 was effective in the volume of 4000 ml and the bacterial strain 99 inhibited in 8000 ml. The general inhibition volume could be considered as somewhere between these values. If only one volume is shown, the effective volumes were the same for both bacterial strains.

Juices N+V

When analyzing the agglutination effect, the juices A, B and C had similar results (Table 12). The products with maximal concentration of PYLOS had 100% inhibition diluted to a volume of 1 L. In comparison with the control, which had a corresponding effect in 1.2-2.3 L,

they were less effective. The control did also have 50% inhibition at a bigger volume than the juices.

The agglutination effect was significant higher in juice D and E. At 100% inhibition, the juice D, with maximal concentration was effective in 4-8 L in comparison to the control that was effective in 1.1-2.3 L. At 50% inhibition, the juice was effective in 32-64 L and the control in 19-38 L. The juice E with maximal concentration of PYLOS had 100% inhibition effect in a volume of 8-16 L and a 50% inhibition in 32-128 L. The activity of the control was unfortunately very low in this experiment and the difference between the product and control should probably not be as big as the results showed.

The agglutination effect against *H. pylori* was decreasing with decreasing amount PYLOS added. At the half concentration (8 + 8 g), the inhibition was effective in about half the volume. The difference was expressed in either 100% inhibition, in 50% inhibition or in both columns. At a quarter of the concentration (4 + 4 g), the effective volume was shown to be a quarter of the volume for maximal concentration, etc.

Table 12 The table shows results from the hemagglutination assay of juices with PYLOS substrate N+V

Juice (250ml)	PYLOS substrate N+V (g)	100% inhib.[15] Sample (ml) Hp.[8] 74 - 99	100% inhib.[15] Control (ml) Hp.[8] 74-99	> 50% inhib.[15] Sample (ml) Hp.[8]74-99	> 50% inhib.[15] Control (ml) Hp.[8] 74-99
A	16+16	1000	1150-2300	4000	19000-38100
A	8+8	500	550-1150	2000	9500-19000
A	4+4	250	300-550	1000	4800-9600
A	2+2	<250	100-300	1000	2300-4700
B	16+16	1000	1150	4000-8000	1150-9500
B	8+8	500	550	2000-4000	550-4700
B	4+4	250	300	1000-2000	300-2400
B	2+2	<250	<100	1000	100-1150
C	16+16	1000	1150-2300	4000	19000-38100
C	8+8	500-1000	550-1150	2000	9500-19000
C	4+4	250	300-550	1000	4800-9600
C	2+2	<250	100-300	1000	2300-4700
D	16+16	4000-8000	1150-2300	32000-64000	19000-38100
D	8+8	4000	550-1150	16000-32000	9500-19000
D	4+4	2000-4000	300-550	8000-16000	4800-9600
D	2+2	2000	100-300	4000-16000	2300-4700
E	16+16	8000-16000	1150	32000-128000	9500-4700
E	8+8	4000-16000	550	32000-64000	4700-2300
E	4+4	4000-8000	300	16000-64000	2400-1150
E	2+2	2000-8000	<100	8000-32000	1150-550
F	16+16	2000-8000	1150	16000-32000	2300
F	8+8	2000-8000	550	8000-16000	1150
F	4+4	1000-8000	300	4000-16000	550
F	2+2	1000-8000	<100	4000-8000	300

Hp[8] = *H. pylori,* Inhib.[15] = Inhibiting

Juices N+FV

The juice A, B and C containing N+FV with maximal concentration of PYLOS showed at least twice as high anti-agglutination effect as the same juices with N+V added (Table 13). Inhibition of 100% efficiency was observed in a volume of 2-8 L and 50% inhibition was observed between 4-256 L. *H. pylori* 99 is as mentioned before, known to have a weaker agglutination activity.

The juice product D had 100% inhibition in a volume of 8-32 L in comparison to 3.4-13.5 L in the control. The 50% inhibition was shown in 256-512 L, a volume as big as 1-2 bathtubs, in comparison with the control that was effective in 27-54 L. The activity was decreasing exponentially with lower amount of PYLOS added. The control used in comparison with product E was unfortunately lower than normal.

Table 13 The table shows results from the hemagglutination assay of juices with Pylos substrate N+FV

Juice (250ml)	PYLOS substrate N+FV (g)	100% inhib.[15] Sample (ml) Hp.[8] 74-99	100% inhib.[15] Control (ml) Hp.[8] 74-99	> 50% inhib.[15] Sample (ml) Hp.[8] 74-99	> 50% inhib.[15] Control (ml) Hp.[8] 74-99
A	16+3.2	2000-8000	3350-13450	4000-256000	13450-54150
A	8+1.6	1000-4000	1600-6650	2000-128000	13450-27000
A	4+0.8	500-2000	350-800	2000-8000	3350-13450
A	2+0.4	500-1000	100-350	1000-4000	1600-6650
B	16+3.2	2000-8000	3350-13450	8000-256000	27000-54150
B	8+1.6	1000-4000	1600-6650	8000-128000	13450-27000
B	4+0.8	500-2000	350-800	2000-8000	3350-13450
B	2+0.4	500-500	100-350	1000-4000	1600-6650
C	16+3.2	2000-8000	3350-13450	16000-256000	13450-54150
C	8+1.6	2000-2000	1600-6650	4000-128000	6650-27000
C	4+0.8	1000-1000	350-800	2000-8000	3350-13450
C	2+0.4	1000-500	100-350	1000-4000	1600-6650
D	16+3.2	8000-32000	3350-13450	256000-512000	27000-54150
D	8+1.6	4000-16000	1600-6650	128000-256000	13450-27000
D	4+0.8	2000-4000	350-350	8000-16000	3350
D	2+0.4	1000-2000	<100-100	2000-8000	1600
E	16+3.2	16000-32000	1600-3350	32000-256000	13450-54150
E	8+1.6	8000-16000	800-1600	32000-128000	6650-27000
E	4+0.8	2000-1000	350	8000	800
E	2+0.4	1000-1000	<100	4000-2000	350

Hp[8] = *H. pylori,* Inhib.[15] = Inhibiting

Fermented milk products, N+V

The agglutination activity of the fermented milk products was very much lower than in the juice products (Table 14). Volumes below 250 ml (<250) means that the product was not effective in the dilution 1:1. The volume in which the fermented milk product H with the maximal concentration acts 100% inhibitive was 0.5-1 L in comparison to 2.3 L in the control. The volume in where the product was 50% effective was not bigger than the 100% volume. The agglutination of *H. pylori* had started already in the dilution 1:2. Product J was shown to have the best results of the fermented milk products with a 50% inhibition in 2-8 L. The fermented milk products had about half the activity as the juice products A, B and C with the same PYLOS substrate N+V.

Table 14 The table shows the results from the hemagglutination assay of fermented milk products with PYLOS substrate N+V

Fermented milk (250ml)	PYLOS substrate N+V (g)	100% inhib.[15] Sample (ml) Hp.[8] 74-99	100% inhib.[15] Control (ml) Hp.[8] 74-99	> 50% inhib.[15] Sample (ml) Hp.[8] 74-99	> 50% inhib.[15] Control (ml) Hp.[8] 74-99
G	16+16	500	2300	2000	9500
G	8+8	500	1150	1000	4700
G	4+4	250	550	1000-500	2400
G	2+2	<250	300	500	1150
H	16+16	500-1000	2300	1000-500	9500-38100
H	8+8	500	550	500	4700-19000
H	4+4	500-250	300	500	2400-9600
H	2+2	250	100	500	1150-4700
I	16+16	500	1150	1000	9500-38100
I	8+8	500-250	550	1000-500	4700-19000
I	4+4	250	300-550	500	4800
I	2+2	<250	100-300	500	2300
J	16+16	500-1000	1150-2300	2000-8000	4700-19000
J	8+8	500	550-1150	500	9500
J	4+4	250	300-550	500	4800
J	2+2	<250	100-300	500	2300

Hp[8] = *H. pylori,* Inhib.[15] = Inhibiting

Investigative tests of fermented milk products

When performing the investigating test with product H (PYLOS; 16 g + 16 g) it was observed that the use of different filter papers, incubation times, dilutions and other technical aspects in the procedure had little impact on the anti-agglutination effect (Table 15). The inhibition volume was found to be the same or very similar as the control (the first product H made). When using centrifugation instead of filtration or no extraction at all, the activity increased. However, the big particles present made the sample difficult to analyze and the result has been considered as less reliable. When adding erythrocytes directly into product H, no agglutination occurred, but when adding *H. pylori* to the mixture, agglutination occurred very quickly.

The chemical test that actually resulted in an increased inhibition effect was when adding HCl to the product. The milk proteins caseins, denaturize already at pH 4.6 and could easily be discarded through centrifugation. The product was then neutralized with NaOH before analyze. The 100% effective inhibition of *H. pylori* increased from 0.5 L to 1 L and the volume in which the product is 50% inhibition increased from 1 L to 4 L. No impact from NaCl was observed on the erythrocytes.

Table 15 The table shows the results from the hemagglutination assay of the investigation tests of product H

Method (Product H, 250ml)	PYLOS substrate N+V (g)	100% inhib.[15] Sample (ml) Hp[8] 74	100% inhib.[15] Control (ml) Hp[8] 74	> 50% inhib.[15] Sample (ml) Hp[8] 74	> 50% inhib.[15] Control (ml) Hp[8] 74
Product H, 0.5%	16+16	500	500	1000	1000
Product H, 0.5% (new test)	16+16	500	500	500	1000
Filter (113 µm)	16+16	500	500	500	1000
Filter (thick)	16+16	500	500	1000	1000
Filter (thinn)	16+16	500	500	1000	1000
Centrifugation (3x4.0 rpm)	16+16	250	500	2000	1000
Dilut 2 times, 4h (thinn filter)	16+16	1000	500	1000	1000
Dilut. 2 times extr. over night	16+16	1000	500	1000	1000
Not extracted	16+16	250	500	2000	1000
Exp 1:1 Change of conc.	16+16	500	500	500	1000
Exp 1:2 Change of conc.	8+8	250	500	500	1000
Exp 1:3 Change of conc.	4+4	<250	500	250	1000
Exp 2:1 Incub. time, 0.5 h	16+16	500	500	500	1000
Exp 2:2 Incub. time, 4 h	16+16	250	500	500	1000
Exp 2:3 Incub. time, o.n.	16+16	250	500	500	1000
3 M HCl added	16+16	1000	500	4000	1000

[7]Conc = Concentration, [8]Hp = *H. pylori*, [16]Incub. time = Incubation time, [17]o.n. = Over night, [18]extr = extracted, [20]Exp = Experiment

Heat treatment

Heat treatment at 72°C for 15 s of milk with the PYLOS substrate N+V was shown to have a small negative effect on the 100% adhesion inhibition against *H. pylori*. The treatment showed a small negative impact of 50% inhibition observing *H. pylori* strain 74, but did not affect the inhibition against strain 99. The same temperature treatment of milk with substrate N+FV had a small negative effect of the 100% inhibition of strain 74, but did not affect the inhibition of strain 99. No effect was observed at the volume of 50% inhibition (Table 16).

Heat treatment at 85°C for 5 s of milk with the PYLOS substrate N+V had a negative effect of the 100% inhibition of *H. pylori* strain 74, but did not affect the inhibition of strain 99. The heat treatment reduced the volume of 50% inhibition to the half. Heat treatment at 85°C of milk with the PYLOS substrate N+FV affected the inhibition of *H. pylori* strain 74 negatively, but did not affect the inhibition of strain 99. The heat treatment did not affect the 50% inhibition volume.

Milk with substrate N+V actually got an improved 100% inhibition effect after heat treatment at 95°C for 10 min. At 50 % inhibition, the heat treatment had a negative effect on inhibition of strain 74, but did not affect the inhibition of strain 99. The result was similar for the milk with N+FV substrate; the 100% inhibition was improved by the heat treatment. The 50% inhibition was negatively affected observing strain 74, but did not change the inhibition against strain 99.

The juice C containing N+V was not observed to have changes caused by heat treatment on the 100% inhibition volume. The volume of 50% inhibition was not changed observing strain 74, but decreased for strain 99. The inhibition activity in the juice containing N+FV was not observed to be affected by the heat treatment.

Table 16 The table shows the results of the hemagglutination assay of the heat treated products

Product and heat treatment (250 ml)	PYLOS substrate	PYLOS substrate (g)	100% inhib.[15] Sample (ml) Hp[8] 74-99	100% inhib.[15] Control (ml) Hp[8] 74-99	> 50% inhib.[15] Sample (ml) Hp[8] 74-99	> 50% inhib.[15] Control (ml) Hp[8] 74-99
Milk, 72°C, 15 s	N+V	16+16	<250	250	500-1000	1000
Milk, 85°C, 5 s	N+V	16+16	<250-250	250	500	1000
Milk, 95°C, 10 min	N+V	16+16	500	250	500-1000	1000
Milk, 72°C, 15 s	N+FV	16+16	<250-250	250	500	500
Milk, 85°C, 5 s	N+FV	16+16	<250-250	250	500	500
Milk, 95°C, 10 min	N+FV	16+16	250-500	250	500-1000	500
Juice C, 90°C, 10 s	N+V	16+16	1000-2000	1000-2000	8000-4000	8000
Juice C, 90°C, 10 s	N+FV	16+16	2000	2000	16000-8000	16000-8000

[8]Hp = *H. pylori*, [15]Inhib = Inhibition

4.2.2. Growth assay

The growth assay was performed on a limited number of products. Table 17-19 shows the volume in which the product with maximal concentration of PYLOS substrate inhibits *H. pylori* growth.

Juices N+V

The inhibition of *H. pylori* growth (Table 17) was less effective compared to the inhibition against bacterial adhesion which was observed in the hemagglutination assay. The juice products A, B and C had similar 100% inhibition activity, a volume that was less than half as big as for the control. The effective volume for 50% inhibition almost corresponded to the control. The juice products D and E almost corresponded to the controls for 100% inhibition and showed a higher efficiency than the controls for 50% inhibition.

Table 17 The table shows the results of the growth assay of juices containing PYLOS substrate N+V

Juice (250 ml)	PYLOS substrate N+V (g)	100% inhib.[15] Sample (ml) Hp[8] 74-99	100% inhib.[15] Control (ml) Hp[8] 74-99	> 50% inhib.[15] Sample (ml) Hp[8] 74-99	> 50% inhib.[15] Control (ml) Hp[8] 74-99
A	16+16	250	550	1000	1150
B	16+16	250	550	1000	1150
C	16+16	250	550	1000	1150
D	16+16	500	550	2000	1150
E	16+16	500	550	2000	1150

[8]Hp = *H. pylori*, [15]Inhib. = Inhibition

Juices N+FV

The juices with PYLOS substrate N+FV were less effective than juices containing N+V (Table 18). The juices containing N+FV did not reach the 100% inhibition level of the controls. The juice D was the only product with 100% inhibition at the dilution 1:1, against *H. pylori*. The volume for inhibition did not differ between the bacterial strains 74 and 99. Juice D and E reached a higher 50% inhibition volume than their controls.

Table 18 The table shows the results from the growth assay of juices containing PYLOS substrate N+FV

Juice (250ml)	PYLOS substrate N+FV (g)	100% inhib.[15] Sample (ml) Hp[8] 74-99	100% inhib.[15] Control (ml) Hp[8] 74-99	> 50% inhib.[15] Sample (ml) Hp[8] 74-99	> 50% inhib.[15] Control (ml) Hp[8] 74-99
A	16+3.2	<250	350	500	800
B	16+3.2	<250	350	500	800
C	16+3.2	<250	350	500	800
D	16+3.2	250	350	1000	800
E	16+3.2	<250	350	1000	800

[8]Hp = *H. pylori*, [15]Inhib. = Inhibition

Fermented milk products

In the fermented milk products, the activity against *H. pylori* growth was observed to be less than half as effective as the controls (Table 19). No difference could be seen between the products. The inhibitory effect in the fermented milk products corresponded to the effect of some of the juices with PYLOS substrate N+FV.

Table 19 The table shows the results of the growth assay of fermented milk products with Pylos substrate N+V

Fermented milk (250ml)	PYLOS substrate N+V (g)	100% inhib.[15] Sample (ml) Hp[8] 74-99	100% inhib.[15] Control (ml) Hp[8] 74-99	> 50% inhib.[15] Sample (ml) Hp[8] 74-99	> 50% inhib.[15] Control (ml) Hp[8] 74-99
G	16+16	<250	550	500	1150
H	16+16	<250	550	500	1150
I	16+16	<250	550	500	1150
J	16+16	<250	550	500	1150

[8]Hp = *H. pylori*, [15]Inhib = Inhibition

Heat treatment

In the heat treated products, no difference could be seen between the products and the controls at 100% inhibition (Table 20). At 50% inhibition, milk heat treated at 85°C for 5 s and at 95°C for 10 min showed about half the efficiency than the controls. There were no difference found between the heat treated juices and the controls.

Table 20 The table shows the results of the growth assay of the heat treated products

Product and heat treatment (250)	PYLOS Substrate	PYLOS Substrate (g)	100% inhib[15] Sample (ml) Hp[8] 74-99	100% inhib[15] Control (ml) Hp[8] 74-99	> 50% inhib[15] Sample (ml) Hp[8] 74-99	> 50% inhib[15] Control (ml) Hp[8] 74-99
Milk,72°C, 15 s	N+V	16+16	<250	<250	1000	1000
Milk, 85°C, 5 s	N+V	16+16	<250	<250	500	1000
Milk, 95°C,10 s	N+V	16+16	<250	<250	500	1000
Juice C, 90°C, 10 s	N+V	16+16	250-<250	250	1000	1000
Juice C, 90°C, 10 s	N+FV	16+3.2	<250	<250	500	500

[8]Hp = *H. pylori*, [15]Inhib. = Inhibition

5. DISCUSSION

5.1. Product development

The results of the sensory test of the products developed showed that the juices containing PYLOS substrate N+FV got the best sensory profiles both in terms of taste and consistency. Using FV instead of V decreased the taste of bitterness and also improved the mouth feeling and the smoothness of the juice. The walnut substrate was not beneficial for the colour in none of the juices, but for juice E, where no colour change was observed. The amount of substrate added had a large impact of the taste and consistency. The best general grade was found in those juices with lower amounts of PYLOS substrate. Generally there was the walnut substrate that caused an odd taste in the products.

The fermented milk products got good grades in the sensory test. The taste of rosehip and walnut fitted well together with the fermented milk and any bitterness was not noticeable. The products had a very pleasant consistency and the grades increased with the amount of PYLOS added. A potential addition of sugar to the product or the taste of vanilla would probably increase the positive eating experience further more.

5.2. Verifying microbiological analyses

The verifying microbiological tests showed that the juice products D and E were highly inhibitory against *H. pylori*. The juices were shown to be more effective than their controls, which indicates that the main fruit component in these juices enhance the inhibition effect of the PYLOS substrate. Most effective was the juice E with PYLOS substrate N+FV. The active chemical component of PYLOS which inhibits *H. pylori* is not known, but the inhibition effect has been reported also from other plants than rosehip and walnut. The second main component in juice E has earlier been reported to have activities against the bacteria. High molecular weight polysaccharides originating from the seeds of these berries have been described to inhibit adhesion of *H. pylori* to the gastric mucosa *in vitro* (Galan, *et*

al., 2004). Other fruit and berries have also been shown to have similar effects (Miller-Podraza, 2009).

The PYLOS substrate N+FV was shown to have a stronger inhibition effect against bacterial adhesion than N+V. This does not support the general view that whole particles of walnut would be more effective, due to more components present and no activity loss in the extraction stage. A reason could be that the used weight relationship between V and FV was not in balance. The freeze dried walnut extract had most likely a higher activity/g than presumed. The PYLOS substrate N+V was however more effective against growth of *H. pylori*. In a health perspective, N+V might be more likely to use due to its higher content of fatty acids and bigger particles which is present in the stomach for a longer time.

A problem regarding natural plant products in a functional food aspect is that the components and the activity might vary between the seasons. Many factors as weather, cultivation site, harvest, degree of maturity and storage conditions can influence the anti-microbial activity of the product. Because of this issue, supplier assurance has to be done to declare that the activity meets a certain lower limit.

Treatment of controls

The controls used were extracts of N+V and N+FV for products with N+V and N+FV, respectively. The same control samples were used for all products and were kept in a freezer between usages. The effects of the controls varied unfortunately from the beginning of the analyzing period to the last usage. In some analyses, the controls had a lower inhibition effect than usual. The reason was probably that freezing the control sample several times affected the activity negatively. The controls should have been kept frozen in smaller batches to avoid the activity loss during several thawing and refreezing procedures.

Investigation tests of fermented milk products

When *H. pylori* attach to cells in the gastric mucosa, interactions occurs between bacterial carbohydrate binding proteins, lectins, and glycoconjugates on the surface of the cell. Selected sialic acid binding proteins are the group of lectin adhesines that are important in the establishment of the colonization in this infection process (Miller-Podraza, 2009). When *H. pylori* was added to the fermented milk products, a rapid agglutination was observed and the fermented milk products were shown to be less effective compared to the juice products.

Milk proteins are separated into caseins and whey proteins. Of the casein proteins, κ-casein (kappa-casein) differs from the others. Among others it has two cysteine residues that have the possibility to form disulfide bonds. Further, about two thirds of the monomeric molecules contain a carbohydrate group which is esterified to one of the threonines amino acids and has galactosamine, galactose and one or two sialylated structures containing NeuAc (N-acetylneuraminic acid) epitopes (Walstra, *et al.*, 2006). Κ-casein is the milk protein that contains significant amount of sialic acid (Marier, *et al.*, 2010). It is theoretically possible that the presence of sialylated oligosaccharides and sialylated glycoproteins has interfered with the hemagglutination-inhibition tests. The sialylated milk compounds thus competed with

sialylated saccharides present on erythrocytes resulting in several binding sites for the bacteria, which led to faster agglutination and a weaker inhibition effect of PYLOS.

Centrifugation of a fermented milk product

In the investigation tests, hydrochloric acid (HCl) was added to one fermented milk product to denature the proteins. However the result of this test had been the same without adding HCl since the isoelectric point of caseins are about pH 4.6 (Walstra, *et al.*, 2006). In fermented milk products, the lactic acid bacteria have lowered the pH to about 4.5 and therefore the caseins are uncharged and precipitated. Centrifugation thus removed all caseins and the supernatant without κ-casein and sialic acid was neutralized and used for hemagglutination. When *H. pylori* then was added to the product, less extra sialic acid bounding sites was present and the inhibition effect of PYLOS was increased.

Heat treatment

When milk is heated to 70-90°C, globular proteins as β-lactoglobulin, α-lactalbumin, serum albumin and immunoglobulins are denatured. Under that process they exhibit unfolding of their peptide chains and mostly lose their biological activity as an enzyme or an antibody and become less soluble. When heating, the whey protein β-lactoglobulin exposes a free thiol group, which reacts with other disulfide groups and form dimers. Further aggregation may occur and trimers and tetramers are produced (Walstra, *et al.*, 2006). In contrast to the whey proteins, the caseins proteins are not heat denatured. The denatured serum proteins associate with the micelles through disulfide bonds at the casein micelle surface (Andrén, 2011). When heating the milk at 95°C for 10 min as in the heat treatment for the process to produce fermented products, the ability of *H. pylori* to attach to the sialic acids on the κ-casein might have decreased because of the steric hindrance that appears when the serum proteins are covering the micelle surface. The effect of agglutination might therefore have been increased and that was also shown via similar results of fermented products and milk heat treated at 95°C for 10 min.

The heat treatment of milk inhibited the reaction between *H. pylori* and the sialic acid glycosylated to the κ-casein, but it also seemed to affect the agglutination inhibition of the PYLOS substrate negatively. If the results in Table 16 are compared, lower temperatures decreased the function of the PYLOS substrate, while heat treatment at higher temperatures inhibited the bacterial binding to κ-casein, which resulted in a net positive outcome. Most likely a higher temperature also affects PYLOS negatively. The substrate N+V seemed to be more sensitive for heat treatment than N+FV. It means that the powder substrate of walnut was affected to a greater extent of heat treatment than the freeze dried extract.

Differences between H. pylori 74 and 99

The inhibition volume was generally lower for the *H. pylori* strain 74. That means that this strain had a stronger agglutination effect and was less affected by the inhibition substrate. *H. pylori* strain 99 had a weaker agglutination and gave therefore better inhibition results. Both strains are however capable to cause infection in the gastric mucosa and the effects of the

PYLOS products could be expected to have a practical effect in-between the inhibition volumes for *H. pylori* 74 and *H. pylori* 99. The inoculation of the bacteria was done with the same procedure every day. There is thus an option that the amount of bacteria used might have differed among the many analysis performed.

Method evaluation

The growth assays confirmed the results of the hemagglutination assays. The volume in which PYLOS was effective was smaller, but the results showed the same trend. When watching the results for the growth assay, it is important to consider that when 200 µl of the product with PYLOS substrate was added to the agar gel, an unknown amount was diffusing into the gel of 3 ml. The dilution of the product was therefore greater than specified in this report, which could explain the lower effective volumes in the results for the growth assays.

This report showed *H. pylori* inhibition at 50% as the lowest level. Effects at 25% and 15% inhibition were not taken into account and therefore the resolution of the results could have been better if lower inhibition effects also were considered. For example, two products with different concentrations of PYLOS substrate could have the same effect at 50% inhibition, but differ remarkably at 25% inhibition.

It is also important to consider that these experiments included many technological steps, not occurring in the stomach, which could have decreased the product effect. One technical aspect decreasing the PYLOS effect was for example the filtration process, where much of the substrate was caught in the filter. The active compounds could therefore have been highly reduced and not used in the verifying microbiological analysis. Therefore it is likely to presume that the inhibition effect of the PYLOS substrate is higher when acting *in vivo* than *in vitro.*

The recommended product

The juice product E with PYLOS substrate N+FV had satisfactory results in both the sensory test and the verifying microbiological analyses. The product had a pleasant taste with prominent flavors of the main fruit components. The bitterness of walnut was hid to a higher extent. The colour was purple and gave the juice a nice visually appealing. The test panel accepted the product taste even at higher concentrations of PYLOS substrate.

In the hemagglutination tests, juice E was shown to have an impressing inhibition effect against *H. pylori* and almost all concentrations tested had better results than the controls, which indicated that main components enhanced the effect of PYLOS. A product with maximal concentration of PYLOS substrate in a volume of 250 ml was 100% effective against *H. pylori* adhesion in a volume of 16-32 L and 50% effective in 32-256 L. The product was 100% effective against *H. pylori* growth in 0.25 L and 50% effective in 1 L.

The aim of this future product is not to totally eliminate *H. pylori*, but to reduce the number of bacteria to decrease problems with gastric ulcer and to prevent stomach cancer caused by

prolonged infection. There is thus no need to use the maximal concentration of PYLOS substrate added.

6. CONCLUDING REMARKS

The product to be recommended as preferable for addition of the PYLOS substrate, is the juice product E with the PYLOS substrate N+FV.

Fermented products are not suitable for this purpose due to the presence of sialylated compounds in milk, although fermented products with PYLOS substrate received high scores in the sensory test.

To continue this project

This report have shown which products from the product range of Skånemejerier that would be preferable to use in the context of including PYLOS to a food product, in purpose to inhibit *H. pylori.*

To continue this work, the juice product E containing PYLOS substrate N+FV is being recommended as a product to analyze further. It would be desirable to get statistical significance of the result based on several analyses and statistical calculations. To see the practical use of this food in a new clinical study would also be interesting for further knowledge and one step towards product integration to the market of functional foods.

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