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COMBINING BIOCHEMISTRY TO DENTISTRY: FROM *IN VITRO CANDIDA GLABRATA* OBSERVATIONS TO AN *IN VIVO* CLINICAL LINGONBERRY APPLICATION

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Biological
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Cover: *C. glabrata* disc diffusion assay: 50x concentrated FLJ (left);
0,2% chlorhexidine (right).

CONTENTS

1 ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (referred in the text by Roman numerals (I- IV).

I   Pärnänen, P., Meurman, JHM., Nikula-Ijäs, P. A novel *Candida glabrata* cell wall associated serine protease. Biochemical and Biophysical Research Communications 2015; 457: 676-680. https://doi.org/10.1016/j.bbrc.2015.01.047.

II   Pärnänen, P.; Nawaz, A.; Sorsa, T.; Meurman, J.; Nikula-Ijäs, P. The Effect of Fermented Lingonberry Juice on *Candida glabrata* Intracellular Protein Expression. International Journal of Dentistry 2017; 6185395. https://doi.org/10.1155/2017/6185395.

III   Hoornstra, D., Vesterlin, J., Pärnänen, P., Al-Samadi, A., Zlotogorski-Hurvitz, A., Vered, M, & Salo, T. Fermented lingonberry juice inhibits oral tongue squamous cell carcinoma invasion *in vitro* similarly to curcumin. In Vivo 2018; 32: 1089-1095. https://doi.org/10.21873/invivo.11350.

IV   Pärnänen, P., Nikula-Ijäs, P., Sorsa, T. Antimicrobial and anti-inflammatory lingonberry mouthwash- a clinical pilot study in the oral cavity. Microorganisms 2019; 7:331. doi: 10.3390/microorganisms7090331.

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2   ABBREVIATIONS

A. a	*Aggregatibacter actinomycetemcomitans*
AGE	advanced glycation endproduct
APMA	aminophenylmercuric acetate
BOP	bleeding on probing
C. albicans	*Candida albicans*
C. glabrata	*Candida glabrata*
C. krusei	*Candida krusei*
C. parapsilosis	*Candida parapsilosis*
CWP	cell wall protein
2D-DIGE	two- dimensional difference gel electrophoresis
DTT	1,4- dithiothreitol
E-Cad	epithelial cadherin
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FLJ	fermented lingonberry juice
F. nucleatum	*Fusobacterium nucleatum*
GPI	glycosylphosphatidylinositol
IL-1β	interleukin-1-beta
LC-MS/MS	liquid chromatography tandem- mass spectrometry
MDPF	2- methoxy- 2,4- diphenyl (2H)- furanone
MMP-8	matrix metalloprotease-8
NAC	non- *albicans Candida*
NET	neutrophil extracellular trap
OTSCC	oral tongue squamous cell carcinoma
PIR proteins	proteins with internal repeats
PMSF	phenylmethylsulphonyl fluoride
P. gingivalis	*Porphyromonas gingivalis*
Sap	secreted aspartyl protease
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
S. mutans	*Streptococcus mutans*
TIMP	tissue inhibitor of metalloprotease
TNF-α	tumour necrosis factor alpha
VPI	visible plaque index
YPG	yeast peptone glucose

3 ABSTRACT

Our studies focused on using *Candida glabrata* (*C. glabrata*) as a model organism to isolate and investigate the role of *C. glabrata* cell wall proteases as host protein-degrading virulence factors and the inhibition of their action. The cell wall proteins of microbes are in the frontline of first contact with the host cells in oral mucosa. *C. glabrata* is the second most prominent *Candida* yeast, and it is commonly found in the normal oral microbial flora causing opportunistic yeast infections, particularly in hospitalized patients. It is considered innately azole- resistant and treatment is more difficult compared to the typical *Candida albicans* (*C. albicans*) infections. Azoles are the most commonly used antifungal agents used in candidosis. There is an urgent need of development of topical antimicrobial agents, and wild berries such as lingonberry, have been increasingly studied. Lingonberries are known to have antioxidant, anti-inflammatory, antimicrobial and anticancerous properties and are considered beneficial to health. To this background we studied *in vitro* and *in vivo* the effects of a patented, fermented lingonberry juice (Lingora®, from now on abbreviated as FLJ). It was specially developed to be used as a mouthwash on *C. glabrata* and other typical microbes of the oral flora related to yeast infections and caries.

Our primary goal was to isolate, identify and characterize *C. glabrata* cell wall proteases with biochemical methods: enzymatic treatment of *C. glabrata* cells, MDPF-zymography, SDS-PAGE, 2D-PAGE and LC-MS/MS. These methods may be used to isolate and identify novel *Candida* cell wall proteases enabling their further characterization and inhibition studies. Further *in vitro* studies were conducted on the effect of FLJ on intracellular protein expression of *C. glabrata* with the 2D-DIGE method. The proteins were identified by LC-MS/MS. The inhibition of proliferation and invasion of two aggressive oral tongue squamous cell carcinoma (OTSCC) lines (HSC-3, SCC-25) with FLJ and curcumin were measured *in vitro* by colorimetric ELISA and three- dimensional Myogel spheroid assay. Finally, we conducted a clinical pilot study including oral examinations, microbial cultivations and measurements of active MMP-8 concentrations using PerioSafe® point-of-care test. FLJ was used as a mouthwash to see if it has also *in vivo* effects on three microbes of the oral microbiota.

From the *C. glabrata* cell wall we identified a novel, uncharacterized 25 kDa serine protease, Cwp1.2., with an estimated pI of 7.6 and gelatinolytic activity. This activity was inhibited by PMSF, a known serine protease inhibitor. Certain *C. glabrata* intracellular protein expressions related to glycolysis, oxidative phosphorylation, oxidative stress and biofilm formation were significantly diminished after treatment with FLJ. These proteins include e.g. heat shock protein and redoxin, which are

expressed by *C. glabrata* when predisposed to stress. Downregulation of these proteins causes *C. glabrata* cells to be more vulnerable to environmental stress and may cause lower virulence. FLJ showed to inhibit proliferation and invasion of two aggressive OTSCC cell lines similar to curcumin. FLJ is safe, has no known interactions with medications and could be studied to be used as an adjunctive therapy in management of OTSCC. The clinical mouthwash pilot study with FLJ results showed statistically significant reduction in *Candida* and *S. mutans* counts. Our *in vitro* studies also indicate growth inhibitory effect on the most common periodontitis- related bacteria. Bleeding on probing (BOP), visible plaque index (VPI) and trend of active matrix metalloprotease-8 (aMMP-8) values were also reduced during the FLJ mouthwash period. Lactobacilli counts increased during the mouthwash period. Although lactobacilli are thought to be related to caries the clinical parameters and clinical outcome indicate a balancing effect on the oral microbial flora from a dysbiotic to a symbiotic direction. This diminished microbial related inflammatory burden should be studied further in context with broader positive general health effects. The results show several beneficial aspects of FLJ in the oral environment. The methodology used in these studies might be applicable to other oral microbes in developing novel antimicrobial agents related to cell wall proteases of *Candida*. Combined *in vitro* and *in vivo* studies showed effects of FLJ on *C. glabrata* intracellular proteins, host cell derived proteins including anti-inflammatory effects, tongue carcinoma cells and oral microbiota.

4   INTRODUCTION

The oral cavity harbours approximately 600- 700 microbial species, including bacteria, yeasts and viruses, of 50% cannot be cultured. In oral health these microbes exist as commensals, not producing harm to the host (Aas et al. 2005, Dewhirst et al. 2010). The microbial flora may harbor also opportunistic pathogens, which may cause infections if the host immune response is out of balance. Clinically relevant bacterial species include *Streptococci* (e.g. *S. mutans*), which are related to dental caries; periodontitis- and gingivitis- related species, such as *Spirochaetes, Fusobacteria, Actinobacteria, Firmicutes, Proteobacteria, Bacteroidetes* (Paster et al. 2001, Visser et al. 2011) and *Candida* yeasts which may cause candidosis in the oropharynx. Due to the clinical significance of *C. glabrata* infections and related drug- resistance problems, *C. glabrata* was selected as a model organism to study cell wall associated proteins and their interactions with host proteins - aiming to isolate and characterize *C. glabrata* proteases involved in virulence.

REVIEW OF THE LITERATURE

Candida glabrata as a model organism

C. glabrata is a haploid budding yeast (1-4 µm), occurs as planktonic cells and forms no true hyphae (Fidel et al. 1999). It is genome is phylogenetically closer to *Saccharomyces cerevisiae* than to *C. albicans*. C. *glabrata* belongs to the family of non- *albicans Candida* (NAC), such as *C. tropicalis*, *C. krusei*, *C. dubliniensis* and *C. parapsilosis* (Tam et al. 2015).

   C. glabrata is regarded the second most common yeast which causes mucosal infections in humans, particularly in individuals with predisposing factors such as cancer, diabetes and acquired immune deficiency syndrome (AIDS) (Weig et al. 2004, Li et al. 2007). *C. glabrata* is found in the normal oral flora but it may cause opportunistic, often life- threatening, systemic infections (Pfaller & Diekema 2007). The treatment of candidosis caused by *C. glabrata* is more cumbersome compared to the most prevalent species *C. albicans* (Fidel et al. 1999, Rodrigues et al. 2014), because it is considered increasingly azole- resistant (Vale-Silva & Sanglard 2015) and even echinocandin resistant (Pfaller et al. 2019). Azoles are the most widely used antifungals for treatment of candidosis.

   Antifungal drug tolerance and sugar sensing is an important factor in contact with host immune cells, oxidative stress resistance, antifungal drug tolerance and invasive processes (Van Ende et al. 2019). *C. glabrata* is capable to form biofilms on

surfaces much more efficiently than *C. albicans*. This biofilm formation is enabled by adhesins (Kumar et al. 2019) on the surface of the yeast cell. *Candida* cells attach to the epithelial cells via adhesive proteins. *C. glabrata* has a very high number of adhesin- like GPI- proteins. These proteins are involved in adhesion to host tissue and biofilm formation (de Groot et al. 2008).

Biofilms are more resistant to antifungals and makes the organism more virulent. In the hospital environment biofilm formation of *Candida* species on medical devices or invasive procedures make way for systemic *Candida* infections. In a study by Nunez-Beltran et al. 2017 the adherence of *C. albicans*, *C. glabrata*, *C. krusei* and *C. parapsilosis* to polyurethane, PVC and silicone was evaluated revealing of the involvement of yeast cell wall associated moonlighting proteins in this process. Traditionally yeast cell wall proteins are guided to the cell wall by a signal sequence at the aminoterminal end of the protein. Moonlighting proteins do not have a signal sequence: they are secreted in extracellular vesicles transporting them to the cell wall.

The microbial cell wall sugars and proteins are the first molecules contacting the host epithelial cells in skin and mucosa. The pathogen- specific patterns on microbial cell surfaces initiate host innate immune responses. To avoid immune recognition of *C. albicans* β- glucan is masked, but the immune system unmasks the hidden epitope by a neutrophil extracellular trap (NET) mediated attack remodeling the fungal cell in a way that enhances immune recognition (Hopke et al. 2016). *C. glabrata* has been shown to trigger NET release in a rat vascular *in vitro* model. This NET release is diminished in exposure to *C. glabrata* biofilm compared to planktonic cells (Johnson et al. 2017). *C. glabrata* has certain differences in attachment, obtaining of nutrients an evading the immune response in the infection process compared to *C. albicans* (Brunke & Hube 2013). *C. glabrata* may evade the immune system by persisting and multiplying in macrophages (Seider et al. 2011).

C. glabrata cell wall associated proteases

All yeasts share a basically common cell wall structure, but the composition of constituents varies between species. The cell wall structure is composed of polysaccharides (80% of dry weight; glucans, chitin chitosan), proteins (3- 20% dry weight; O- or N- glycosylated), lipids (1- 10% dry weight) and pigments (Ruiz-Herrera 2012). The general cell wall structure of *C. glabrata* is shown in figure 1A. In figure 1B a detailed composition and glucan- linkages on the cell wall is shown. Cell wall proteins can be divided to three groups: 1) glycosylphosphatidylinositol (GPI) proteins, which are covalently bound to the wall 1,6-β-glucan, 2) proteins with internal repeats (PIR) are linked through a mild-alkali-sensitive linkage to 1,3-β-glucan and 3) proteins attached to the cell wall with a disulphide bond and detached by treatment with β- mercaptoethanol or dithiotreitol (DTT) (de Groot et

al. 2008). GPI-proteins have high serine (Ser) or threonine (Thr) residue amounts and are usually highly O- glycosylated (Ruiz- Herrera 2012).
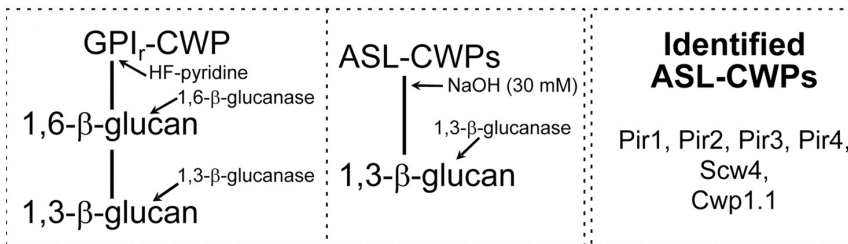
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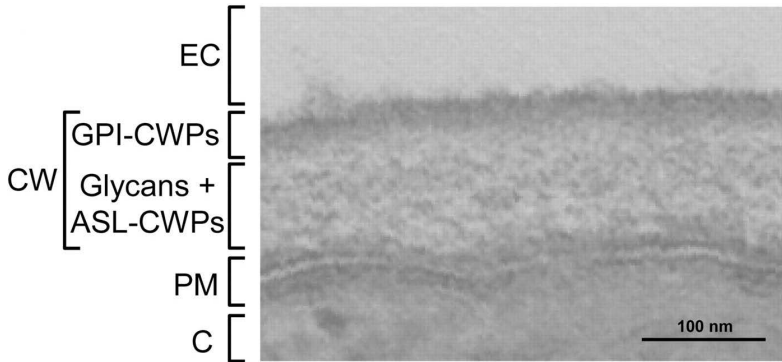
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Figure 1. **A** The general structure of the yeast cell wall. Cell wall (CW); extracellular environment (EC); plasma membrane (PM); cytosol (C); mild-alkali extracts of cell-wall proteins (ASL-CWPs); GPI-modified cell-wall proteins (GPI-CWPs). **B** Identified cross-links between proteins and β-glucans in the cell wall of *C. glabrata* (in the left and middle panels). The extraction methods used to solubilize cell wall proteins (CWPs) are indicated. On the right panel cell wall proteins identified in mild-alkali extracts by LC-MS/MS (ASL-CWPs), proteins with internal repeats (PIR). Modified from: de Groot et al. 2008.

C. glabrata possesses multiple virulence factors e.g.: adhesins, biofilm formation, lipases and proteases (Silva et al. 2012). It does not secrete aspartic proteases (Saps) which are known virulence factors of *C. albicans* and the other NAC species mentioned above (Dostal et al. 2003, Kaur et al. 2005) but has GPI- linked aspartic proteases, yapsins, on the cell surface (Kaur et al. 2007). Moonlighting proteases are intracellular proteins which may also be secreted in a non- traditional or atypical manner, without an endoplasmic reticulum/ Golgi N-terminal signal sequence, in exosomes to the cell wall (Gil-Bona et al. 2014). Moonlighting proteases have more than one biochemical or biophysical function

in one polypeptide chain (Jeffery et al. 1999). They have been found from humans and several bacterial species including oral streptococci and lactobacilli (Delgado et al. 2001, Kainulainen et al. 2012, Giardina et al. 2014), and yeasts *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* (Alloush et al. 1997, Nombela et al. 2006, Ramirez- Quijas et al. 2015, Serrano-Fujarte et al. 2016, Karkowska- Kuleta et al. 2019). Intracellular functions of these enzymes include glycolysis, citric acid cycle, pentose phosphate pathway, nucleotide or amino acid metabolism, transcription regulation, protein synthesis etc. Moonlighting proteins have been shown to take part in binding extracellular matrix proteins, including fibronectin, laminin, and/or collagen and may serve as commensal microbial attachment to the host or virulence factors of pathogens, chaperones or have enzymatic activity (Jeffery 1999, Amblee & Jeffery 2015). Their expression is induced by environmental signals such as sugar depletion, pH changes, oxidative stress (Giardina et al. 2014, Ramirez- Quijas et al. 2015, Serrano-Fujarte et al. 2016).

Host cell proteins and interaction with *Candida*

Structural protein components of oral mucosa

The host oral mucosa is in first contact with colonizing pathogens and it is a barrier against their invasion deeper into the tissue. Mucosal epithelium consists of a stratified squamous cell layer, extracellular matrix and underlying connective tissue and the composition varies depending on location in the mouth. The epithelial cells are connected to each other by epithelial junctions, such as tight junctions/ claudins (Furuse et al. 1998), occludins (Furuse et al. 1994) and zonulin (Wang et al. 2000, Sapone et al. 2006); adherent junctions/ E-cadherin (E-Cad, Chitaev and Troyanovsky 1998) and desmosomes / desmogleins, desmocollin, plakoglobin / γ- catenin and desmoplakin. The length and homogeneity of the desmosome is considered as a criterium for predicting oral squamous cell carcinoma diagnosis and prognosis (Oliveira Crema et al. 2005).

The basement membrane, located under the epithelial layer, contains e.g. laminins, type IV collagen and proteoglycans depending on location in the oral mucosa. Hemidesmosomes attach epithelial cells to the basement membrane via α6β4- integrin (Litjens et al. 2006).

Between the basement membrane and connective tissue is the extracellular matrix (ECM), a fibrous network composed of collagens, elastin, glycosaminoglycans, fibronectin; cells of the defensive system, matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs), which can also be found in sulcular fluid and saliva. The epithelial layer attaches to the underlying tissue with hemidesmosomes.

Under the ECM are connective tissue proteins e.g. collagens (types I-VI), elastin, tenascin, osteonectin, decorin, tenascin and fibronectin. Gelatin used in the thesis studies is denatured non- triple helical collagen. Collagens are abundant components in the connective tissue.

Matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs)

MMPs are a group of proteases involved with ECM remodeling and degradation in oral health and diseased state such as periodontitis (Sorsa et al. 2004, 2006; Hannas et al. 2007; de Morais et al. 2017). TIMP inhibit the conversion of MMPs to their active forms. Maxillofacial bone remodeling is a complex event balancing between bone metabolism and immune response. In periodontitis there is a constant release of both host-derived inflammatory collagenolytic mediators such as cytokines TNF-α and IL-1β, nitric oxide, reactive oxygen species, MMPs and microbial virulence factors (Visser & Ellen 2011, Alvarez et al. 2019). The saliva of patients harbouring non- *albicans Candida* has been shown to have upregulated IL-1β levels (Nawaz et al. 2015). *Tannerella forsythia*, which is associated with severe periodontal disease, membrane- associated serine protease has been shown to degrade both gelatin and type I collagen (Hockensmith et al. 2016). Proteases isolated from cell extracts from potentially periodontopathogenic bacteria *Porphyromonas gingivalis* (*P. gingivalis*) and *Treponema denticola* have been proven to activate human procollagenases and take part in periodontal collagen degradation (Sorsa et al. 1992). MMPs are secreted in inactive form and are activated upon counter of environmental signals, such as oral microbes. MMPs have indeed been shown to be activated by oral bacteria (Sorsa et al. 1992, Okamoto et al. 1997, Hockensmith et al. 2016, Nieminen et al. 2017). In this process inactive MMP-8 is activated and this conversion can be detected as fragmenting of the enzyme. *C. parapsilosis* has been shown to convert proMMP-9 to its active form. *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. krusei* was shown to fragment TIMP-1 into its inactive form (Pärnänen et al. 2010). The activated form of MMP-8 can be utilized as biomarker in clinical work concerning periodontal disease (Al-Majid et al. 2018) or even used as chair-side screening of prediabetes at the dental office (Grigoriadis et al. 2019).

Oral carcinoma

Oral squamous cell carcinomas (OSCC) encompass at least 90% of all oral malignancies (Massano et al. 2005). Oral carcinoma is classified by TNM Classification of Malignant Tumors (TNM): T describes the size of the original (primary) tumor and whether it has invaded nearby tissue, N describes nearby (regional) lymph nodes that are involved, M describes distant metastasis (spread of cancer from one part of the body to another).

Oral tongue squamous cell carcinoma (OTSCC) is considered the most lethal and treatment resistant form of OSCC: s with a 5- year crude survival rate of 65% in Scandinavia. Survival rates decrease with age (Annertz et al. 2002). The use of alcohol and tobacco are known risk factors of oral carcinoma (Jerjes et al. 2012). HPV-infection, poor oral hygiene, periodontitis, chronic yeast infection (Bakri et al. 2010), ill-fitting dentures or other mechanical irritation may increase the risk of OTSCC (Bektas-Kayhan et al. 2014, Singhvi et al. 2017). The consumption of fruits and vegetables reduces the risk for oral cancer (Pavia et al. 2006), head and neck cancer (Freedman et al. 2008). Consumption of berries reduce risk for e.g. oral cavity, esophageal, breast and lung cancer (Kristo et al. 2016).

In tissue structural context, intact basement membrane is crucial for mucosal epithelial cell normal function and altered expression or structural alteration of these components may lead to functional disturbances, and even cancer cell invasion. Previous *in vitro* studies from our group have shown direct degradation of host cell extracellular matrix components by *Candida*, and periodontitis- related bacteria: gelatin, human laminin-332, human plasma fibronectin, human epithelial cadherin (E- Cad) and claudin- 4 (Pärnänen et al. 2008, 2009, 2010; Pärnänen & Sorsa 2013; Nawaz et al. 2018). Lactobacilli did not degrade E-Cad. Curcumin, a natural substance, has shown to have inhibiting effects on OTSCC proliferation and invasion (Lee et al. 2015) by decreasing MMP-2 and -9 levels, and modulating E-Cad/ p53 pathway.

Lingonberry

Lingonberry, *Vaccinium vitis idaea* L. (also called mountain cranberry or cowberry) is an evergreen wild brush with edible fruits that grows in Eurasia. Lingonberries have been traditionally used in the Finnish folklore to treat thrush. Consuming of berries, fruits and vegetables are recommended to be used 250 g/ day in a healthy diet (Finnish National Nutrition Council 2014). Although scientific studies show various benefits of consuming berries, nutrition- and health claims are still to be approved by authorities (SITRA, EFSA). The phenolic profile is unique for each berry and surprisingly many (e.g. raspberry, sea buckthorn, cloudberry, bilberry, lingonberry, cranberry, strawberry) have pH values of 2.4- 3.5. Lingonberries are rich in phenolic compounds including flavonoids (anthocyanins, flavonols, flavanols); phenolic acids (benzoic acids), lignans, stilbenes (resveratrol), phenolic polymers (ellagitannins and proanthocyanidins) (Ek et al. 2006, Kylli et al. 2011).

Numerous *in vitro* studies have been conducted with lingonberries, which prove antimicrobial (Ho et al. 2001, Puupponen-Pimiä et al. 2005, Nohynek et al. 2006, Riihinen et al. 2011, 2014) and inhibition of adhesion of bacteria (Toivanen et al. 2010). Lingonberries show antioxidant (Kähkönen et al. 2001, Zheng & Wang 2003, Viljanen et al. 2004, Määttä-Riihinen et al. 2005, Wu & Yen 2005, Leiner

et al. 2006, Kylli et al. 2011, Mane et al. 2011) and anti-inflammatory (Kylli et al. 2011) effects and inhibition of advanced glycation end- product (AGE) formation related to diabetes mellitus (Wu & Yen 2005, Beaulieu et al. 2010, Leduc et al. 2016). Antianticancerous effects have also been shown (Olsson et al. 2004, Wang et al. 2005, McDougall et al. 2008). Bioactive molecules of lingonberry such as anthocyanin cyanidin 3-O-galactoside and flavonols have been found from plasma and shown to be excreted in urine (Lehtonen et al. 2009, 2010). A drug absorption *in vitro* study using Caco-2 cell cultures has shown, that ingestion of lingonberry extract is not expected to alter markedly the permeation of simultaneously used highly permeable drugs (Laitinen et al. 2004). Lingonberries have been found to have also *in vitro* antiviral activity. Lingonberry total extract with methanol has been shown to inhibit replication in coxsackievirus B1 and influenza A/ H3N2, anthocyanin- fraction inhibited influenza virus A/ H3N2 (Nikolaeva- Glomb et al. 2013).

Although lingonberries have been studied quite extensively *in vitro* only a few clinical *in vivo* studies have been conducted. *In vivo* rat studies (Kivimäki et al. 2012, 2014) have shown anti-inflammatory and anti- atherothrombotic effects of lingonberry juice. Inhibition of intestinal tumourigenesis (Misikangas et al. 2007) has been shown in mice. A combined *in vitro/in vivo* human study has shown that lingonberry phenolic compounds retain their biological activity analyzed from ileal samples (Brown et al. 2014). *In vivo* glycaemic effects of lingonberries have been studied by Linderborg et al. (2012) and Törrönen et al. (2012, 2013, 2017). Many human studies have been conducted with ingested berry mixtures. The only clinical human study of the effects on the oral cavity with lingonberry has been to the best of knowledge performed by Pärnänen et al. (2019) and is included in this thesis work (study IV).

Although wild berries are generally regarded beneficial to health, they contain a lot of naturally occurring sugars: saccharose, glucose, fructose etc. In the present thesis work a natural clinical patented topical application was developed (Pärnänen 2017). The sugar content of this fermented lingonberry juice (FLJ) was reduced (Pärnänen et al. 2019) to make it suitable for oral use to aid in oral selfcare. The reduction of sugars is essential for or minimizing sugar availability for oral microbes, such as *Candida* in candidosis and *S. mutans* in dental caries development, which use sugars for growth and virulence.

Fermentation of food and juices is a traditionally used method for conserving. It can be performed by lactic acid fermentation or alcoholic fermentation. In the current thesis studies alcoholic fermentation with *Saccharomyces cerevisiae* was used as a safe and economical means to decrease the sugar content from lingonberry juice. Fermentation also softens the taste perception of lingonberries but does not reduce markedly beneficial phenolic concentrations.

5   HYPOTHESES AND AIMS OF THE STUDIES

C. glabrata was chosen as a model organism for the *in vitro* studies. *C. glabrata* uses virulence factors, such as proteases in infecting and invading host tissues. *C. glabrata* cell wall proteases are poorly characterized. The studies (I, II, III, IV) were based on the hypothesis, that *C. glabrata* cell wall proteases have *in vitro* activities to degrade/ modulate oral host proteins. These activities can be inhibited by a specially formulated FLJ which is rich in phenolic substances and the natural sugar content is reduced. The formulated FLJ may inhibit aggressive tongue cancer cell proliferation and invasiveness. The final hypotheses are that if FLJ shows *in vitro C. glabrata* intracellular proteome effects by reducing expression of proteins involved in it's virulence, it may have also *in vivo* antimicrobial and anti-inflammatory effects.

The aims of the studies were:
- isolation and characterization of novel native *C. glabrata* cell wall proteases
- study the direct effect of FLJ/ pH on *C. glabrata* intracellular proteome
- The proliferation and invasion assays of two aggressive tongue cancer cell lines with FLJ
- *in vivo* clinical pilot study using FLJ as a mouthwash

6   MATERIALS AND METHODS

Materials and methods are described here briefly. For more details see original publications enumerated I-IV.

Study I

A clinical *C. glabrata* blood isolate (T-1639, Helsinki University Hospital) was used in the study. It showed typical gelatinolytic activity when compared to several reference and clinical *C. glabrata* strains. A cell wall fraction was obtained by treating yeast cells with β- 1-3-glucanase. Proteins from non-reducing 2D-SDS-PAGE with gelatinolytic activity on a parallel 2D-MDPF (2- methoxy- 2,4- diphenyl (2H)- furanone)-PAGE were identified with LC-MS/MS and Uniprot protein database comparison.

Study II

C. glabrata T-1639 cells were treated with FLJ. The effect of pH was also tested by incubating cells at pH 7.6 and 3.5. The intracellular proteins were isolated and parallel two- dimensional difference gel electrophoresis (2D-DIGE) and 2D-gels were run. Proteins with > 1.5x reduced expressions were cut from the silver stained 2D- gels and identified using liquid chromatography tandem- mass spectrometry (LC-MS/MS) and the Uniprot protein database.

Study III

The inhibition of proliferation and invasion of two aggressive OTSCC lines (HSC-3, SCC-25) with FLJ and curcumin were measured *in vitro* by colorimetric ELISA and three- dimensional tumour Myogel spheroid assay (Salo et al. 2015). A 5-bromo-2'-deoxyuridine kit was used to screen cell proliferation. The proliferation and invasion assays were performed with 500, 2500 and 5000µg/ ml concentrations for FLJ.

Study IV

FLJ was formulated and used as a mouthwash so that 10 mL equals 26 g lingonberries. The sugar content was reduced 8-fold with a pH of 3. A clinical study of 30 adult participants was performed. 20 participants rinsed the oral cavity with 10mL of FLJ twice daily for two weeks and 10 participants 20 mL twice daily for one week. *S. mutans*, *Candida* and lactobacilli were cultivated at the beginning, after the mouthwash period and after a washout period. At the same timepoints an additional oral mouthrinse was collected for chair-side/point-of-care (POC)-PerioSafe®/OraLyzer®aMMP-8quantitative on-line evaluation and an oral clinical investigation was performed.

7   RESULTS

The main results are shown in table 1. Statistical significance is indicated when relevant. For more details on methods, see original publications I-IV.

Study	Main results	Most prominent significance (statistical when applicable)
I (*in vitro*)	A novel *C. glabrata* cell wall protease was identified having a match to Cwp1.2p. It has a molecular weight of appr. 25 kDa (192 aa) and an estimated pI of 7.6. Gelatinolytic activity was inhibited with PMSF.	The methodology may be used to isolate and characterize other unknown *Candida* cell wall proteases with additional biochemical methods (e.g. ion-exchange chromatography).
II (*in vitro*)	Four intracellular proteins with > 1.5 x reduced expressions were identified from the FLJ treated cells: glyceraldehyde 3-phosphatedehydrogenase-2(GADPH-2), adenylate kinase, redoxin Q6FIU4, heat shock protein 9/12 (HSP 9/12). There were no significant effects of pH on intracellular protein expressions.	FLJ has an inhibitory effect on *C. glabrata* cell growth and stress-related protein expression: this may have effects on biofilm formation and virulence. pH did not have a significant effect on the protein expressions.
III (*in vitro*)	FLJ inhibited aggressive OTSCC cell proliferation and invasion significantly. HSC-3 proliferation HSC-3 invasion SCC-25 proliferation SCC-25 invasion	Inhibitory concentration; p-value 2.5- 5.0 mg/mL; p < 0.023 2.5- 5.0 mg/mL; p < 0.0001 2.5- 5.0 mg/mL; p< 0.0001 2.5- 5.0 mg/mL; ns
IV (*in vivo*)	Mean **S. mutans** and *Candida* counts, visible plaque index (**VPI**) and bleeding on probing (**BOP**) were reduced during the mouthwash period. **Lactobacilli** counts increased during the mouthwash period. The **aMMP-8** mouthrinses showed reduced values in both test groups when compared to the start point. The mouthrinse aMMP-8 reduction correlated with the reductions in microbial counts, VPI and BOP.	p < 0,01% (group 1) p < 0,01% (group 1) p < 0,01% (group 1) p < 0,05% (group 1) p < 0,01% (group 1) Statistically nonsignificant, but diminishing clinical trend seen (despite short period of FLJ use).

8   DISCUSSION

This thesis work contains a series of *in vitro* and *in vivo* studies with the aim of linking biochemical *in vitro C. glabrata* observations to an *in vivo* lingonberry clinical application. Although the chemical composition of lingonberries is well known and multiple studies have been conducted, this thesis work includes the first clinical study of the effect of lingonberries in the oral environment. FLJ was formulated suitable for safe use as an aid in oral home selfcare. *C. glabrata* is an emerging opportunistic pathogen and taken to account its common antifungal resistance and clinical relevance it was a representative microbial model organism of the genus *Candida*. FLJ proved to have *in vitro* inhibitory effect on growth of *C. glabrata* and expression of its stress related intracellular proteins related to virulence. FLJ also proved to have *in vitro* anti-carcinogenic effect on aggressive OTSCC cells. The following paragraphs describe individual studies discussing the results in a larger scope.

In vitro studies

The isolation of *C. glabrata* cell wall associated proteases proved to be a multiphase task. The activities of the proteases were maintained by the methods used in the studies (I, II), enabling biochemical assays. The culturing of *C. glabrata* is relatively simple and its proteins are not easily degraded in biochemical processes. Using *C. glabrata* as a model organism the studies have given a glimpse how microbial cell components could be isolated and used for development of new kinds of antimicrobials. By avoiding harsh denaturing/ extraction methods the *C. glabrata* proteases were kept near to native state. *C. glabrata* cell wall proteases are a series of proteases which have yeast cell metabolic functions and are believed to take part in *Candida* virulence. Many of them have been biochemically and functionally characterized, including their molecular weight, optimum pH for activity, secretion pathway and attachment to the cell wall. They are located on the fungal cell wall, which may form a leading- edge component, directly and focally contacting the host cells or tissue structures.

   The novel *C. glabrata* 25 kDa cell wall serine protease was identified and partially characterized by the methods used (**study I**). It is a traditional cell wall protease, with a signal sequence guiding its location to the cell wall. It showed to be gelatinolytic. The fractioning methods used in this study could be further utilized in characterization of also other *Candida* cell wall proteases and their host protein modulation ability. The gelatinolytic activity of these proteases in physiological pH could promote the invasiveness of the fungus into host tissue and

may cause inflammatory reactions causing host tissue aggravated destruction. It is possible, that these proteases are expressed on the yeast cell wall on demand: the environmental signals may be the trigger for *Candida* virulence- related protease expression and activity. In this regard, the cell wall proteases may not only help the fungus to break through the host tissue barriers via its collagenolytic and E-cad/ fibronectin/ laminin-332 degradation activity, but may also guide and promote the yeast cell adherence to matrix components via enzyme/ substrate interaction. In addition, the fungus CWPs may also, by initiation of breakdown of host tissue ECM/ proMMP-8 induce an inflammatory response in the affected tissue. Noteworthy, the gelatinolytic and fibronectin breakdown products are chemotactic for inflammatory cells, which would increase the production of tissue- destructive MMPs by the host. This process would synergistically, together with the fungal CWPs, degrade the host' s basement membrane and connective tissue barrier. Thus, by blocking the ability of the fungal CWPs to activate host cell proMMPs, FLJ could synergistically inhibit fungal cell mediated breakdown of the host' s basement membrane and ECM barrier, and decrease fungal invasiveness into, and destruction of, the deeper connective tissue of the host mucosa.

The treatment with FLJ did not only dramatically and dose/time- dependently decrease growth of *C. glabrata* cells in liquid culturing but caused also > 1.5-fold decrease in intracellular protein expressions of heat shock protein 9/12, redoxin, glyceraldehyde-3-phosphate dehydrogenase and adenylate kinase. Low pH alone did not affect the expression of *C. glabrata* intracellular protein expressions (**study II**). The diminished expression/ action of these proteins may have an effect by weakening *C. glabrata* stress-related virulence, cell wall integrity, energy metabolism and even invasion.

FLJ revealed to have also *in vitro* anticarcinogenic properties: the statistically significant inhibition of proliferation and invasion of two aggressive tongue cancer cell lines (**study III**). The less aggressive SCC-25 cells were more sensitive to FLJ and curcumin than the more aggressive HSC-3 cells. The results are in concordance with previous studies (Olsson et al. 2004, Wang et al. 2005) and indicate that potential anticancerous molecules from nature are still to be found and further investigated. The three- dimensional Myogel spheroid assay (Salo et al. 2015) is based on human uterus benign leiomyoma tumor tissue and as it closely mimics the human tumour microenvironment of solid cancers it is more accurate than the assays composed of non-human derived components.

Throughout the over ten years of studies a total of 6 yeast species (12 strains, both clinical and laboratory strains including *C. albicans, C. dubliniensis*, *C. glabrata*, *C. krusei* and *C. tropicalis*; Pärnänen 2017), 3 periodontitis-related species (6 strains including *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans* and *P. gingivalis*; Pärnänen et al. 2019), 3 streptococcal species (4 strains including two *S. mutans*, one *S. sanguinis* and one *S. salivarius*; Pärnänen 2017) and two

Lactobacillus strains were tested *in vitro* with FLJ. The inhibition of growth was seen in all microbial strains included in the studies, except lactobacilli. As there are hundreds of microbes present in the oral cavity, most of them uncultivable, presented a challenge for choosing indicator microbes relevant to oral health and disease. *Candida*, *S. mutans* and lactobacilli were chosen because of relatively easy cultivation and relationship to *in vivo* monitoring of oral candidosis and dental caries.

In vivo study

The promising *in vitro* studies led to conducting the *in vivo* **study IV** with FLJ, which showed significant reduction of VPI, BOP and inhibition of growth of *S. mutans* and *Candida*, but not lactobacilli. FLJ exerted also anti-inflammatory effects measured with aMMP-8 mouth rinse samples. Chlorhexidine mouthrinses and gels, which are used in management of periodontal diseases show antimicrobial effects *in vitro* (Collins et al. 2018) and *in vivo* (Richards 2017, Fiorillo 2019). Although they are effective, they are not recommended to be used for long periods of time because of their broad- spectrum antimicrobial properties and adverse side- effects. On the contrary FLJ, which also has been shown to inhibit harmful oral pathogen growth *in vitro* (Pärnänen 2017), reduced visible plaque, BOP and aMMP-8 levels *in vivo* (Pärnänen et al. 2019) is safe and can be used daily without side effects.

The aMMP-8 levels may be monitored with a chair-side oral immunotest (Alassiri et al. 2018). Inhibition of tissue reactive oxygen species generated by the host in response to microbial load (Chapple 1997) could be one plausible mechanism of FLJ, as it is rich in antioxidants. Phenolic compounds, as in FLJ, seem to be promising new kinds of antifungals (Martins et al. 2015). The benefit of FLJ is that it does not inhibit the growth of lactobacilli, which are considered beneficial for general health. The use of antibiotics or prolonged chlorhexidine treatments may cause disturbances in the composition of the oral normal flora. The chosen microbial counts to monitor in study IV (*Candida*, *S. mutans*, lactobacilli) were reflected as positive clinical outcomes. Keeping this in mind, promoting of balanced microbiome is considered beneficial for oral health (Hajishengallis 2015, Kilian et al. 2016).

9   CONCLUSIONS

A novel uncharacterized *C. glabrata* cell wall located protease was identified (**study I**) and its host protein gelatinolytic action was inhibited by a serine protease inhibitor. These results indicate that additional unknown cell wall proteases of *C. glabrata* and other *Candida* species may be isolated in their active forms, characterized and screened for potential antifungal agents.

Four *C. glabrata* intracellular stress and energy related protein expressions were 1.5-fold downregulated by treatment with FLJ (**study II**) and the effect was pH independent. There was also an inhibition of *C. glabrata* cell growth which was dose- and time-dependent. These effects may influence *C. glabrata* biofilm formation, cell wall integrity and virulence.

Study III: FLJ inhibited the proliferation and invasiveness of two aggressive OTSCC cells. The inhibition was significant, dose- dependent and similar to curcumin. Screening of other cancer cell lines are warranted. At a clinical point of view, it is possible to consume safely the recommended 120 g lingonberries daily. Keeping in mind, that cancer cell growth is stimulated by sugars, it is obviously a benefit that formulated FLJ contains 1/8 of sugars compared to whole lingonberries.

Study IV showed statistically significant *in vivo* effects of FLJ on three monitorable and prevalent oral microbial species with oral clinical relevance (*Candida*, *S. mutans*, *lactobacilli*) and a trend of diminished active tissue destructive MMP-8 levels. VPI and BOP were reduced significantly. The *in vitro* and *in vivo* study models indicate the potential of FLJ as a novel kind of antimicrobial/ anti-inflammatory/ antiproteolytic that could be used as a topical, adjunctive agent in oral homecare. Using mouthwash after brushing of teeth 1-2 times daily is in safe limits regarding dental erosion and is outweighed by the positive effects on the clinical outcome. This is the first documented clinical study in the oral cavity conducted with lingonberries and shows for the first time the relationship of its effect on three oral microbial counts, clinical oral parameters and aMMP-8. These effects are to be verified with further placebo- controlled studies including impact on caries or candidosis.

The oral microbiota is a complex entity as is the entire gastrointestinal tract. Maybe the future of combatting opportunistic infections would be gentle preventive balancing of the oral flora, by topical agents of natural source, instead of using broad- spectrum antimicrobials. Lingonberries have proven to possess versatile effects and there are indications of beneficial impact on general health.

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Vantaa 04.05.2020
Pirjo Pärnänen

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