MOLECULAR MECHANISMS OF NON-ANTIBIOTIC DRUGS, ACETAMINOPHEN AND IBUPROFEN AS ANTIBACTERIAL AGENTS AGAINST ENDODONTIC PATHOGENS: AN IN SILICO ANALYSIS

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Abstract

Aim: The aim of the study was to elucidate the molecular process underlying the antimicrobial activity of Acetaminophen (APAP) and Ibuprofen (IB) against Enterococcus Faecalis (E. faecalis), Fusobacterium Nucleatum (F. nucleatum) and Peptostreptococcus Micros (P. micros) using in silico analysis.
Materials and Methods: The STITCH v5.0 database was used for detecting the protein-drug interactions. The functional class and virulence property of the proteins were assessed using VirulentPred and VICMPred. PSORTb v3.0 was used to assess the subcellular localisation of virulent proteins. The epitopes were identified using BepiPred v1.0 Linear Epitope Prediction tool.
Results: APAP and IB were found to interact with proteins involved in cellular processes, metabolism, virulence factors and information and storage. The virulent proteins were located in the cytoplasm and cytoplasmic membrane, which would further add to the effectiveness of these compounds to serve as potential antimicrobial agents. Finally, epitope prediction showed multiple epitopes in the virulent proteins for target specific optimization of antimicrobial agents.
Conclusion: APAP and IB were found to target virulent proteins in cellular processes and metabolism of E. faecalis, F. nucleatum and P. micros with information on specific epitopes for target-aided therapeutics. To confirm the genuine interactions between the drugs and the protein repertoire of pathogens, more in vitro research on a wide range of pathogens are needed.

Keywords: Acetaminophen; Ibuprofen; Enterococcus Faecalis; Fusobacterium Nucleatum; Peptostreptococcus Micros; In Silico.

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1. Introduction

Acetaminophen (paracetamol) and other nonsteroidal anti-inflammatory drugs (NSAIDs), such as diclofenac and ibuprofen are some of the most commonly used drugs. Acetaminophen has antipyretic and analgesic action. NSAIDs also possess significant analgesic, antipyretic and anti-inflammatory action which act through the inhibition of prostaglandin synthesis. In addition to these activities, it has been known, but neglected, for years that antipyretic drugs and NSAIDs also have direct and indirect antimicrobial effects. [1,2]

Dental diseases are most commonly associated with pain which may be linked to infections, for example, dental caries, reversible or irreversible pulpitis, or pain associated with post-treatment. [3] NSAIDs are potent analgesics that are widely being used for pain management. Acetaminophen is a frequently used analgesic as first-line medication for pain relief. [4]NSAIDs are typically well accepted by majorities and the side effects are minor and reversible. When administered in therapeutic doses, acetaminophen is relatively non-toxic. [5]

Any novel drug developed should be versatile in order to counteract a wide range of illnesses caused by emerging infections. In addition to their regular functions, antihistamines, antipsychotics, antipyretics and NSAIDs have been reported to have antibacterial activity. [6,7,8] Antipyretics, at therapeutic plasma levels have shown to inhibit the growth or replication of bacteria and fungi. [1] Ibuprofen demonstrated moderate efficacy to eliminate biofilms of S. aureus. [2] Non-antibiotic medications have sparked attention because of the rise of drug resistant bacteria. [9] In light of the aforementioned considerations, it is critical to maximise the antibacterial potential of these medications.

Antibacterial agents that are routinely used work by inhibiting the cell wall, altering the membrane, inhibiting nucleic acid synthesis, and inhibiting protein synthesis. [10] Although various studies have shown the significance of acetaminophen and Ibuprofen as antibacterial agents, the major void is that the underlying molecular processes as to how these drugs target the organism remain unknown. Endodontic infection causes inflammatory reaction in the periapical tissues which is induced by microbial infection. [11] Gaining a better understanding of the pathways targeted by non-antibiotic medications will help repurpose these treatments to treat a wider range of illnesses including antibiotic-resistant strains. Hence, the present study was aimed to unravel the molecular mechanisms underlying the antibacterial activity of acetaminophen (APAP) and ibuprofen (IB) on endodontic pathogens - Enterococcus Faecalis (E. faecalis), Fusobacterium Nucleatum (F. nucleatum) and Peptostreptococcus Micros (P. micros) which are considered to play a pivotal role in endodontic infection.

2. Methodology

Study design

Approval from the ethical committee was taken before starting the study. SRB number - SRB/SDC/ENDO-2102/21/035. The current study followed an observational study design with the goal of identifying the virulence factors or proteins of E. faecalis, F. nucleatum and P. micros which possibly interact with APAP and IB. STITCH v.5 pipeline [12] was used to evaluate the drug interactions with the proteome of bacteria. The virulence properties of the interacting proteins were determined by VICMPred [13] and VirulentPred [14] softwares. Enterococcus Faecalis V583, Fusobacterium NucleatumATCC 10953 and Parvimonas Micra ATCC 33270 were the strains used in the present study.

Protein-drug interactions prediction

STITCH database (Version 5; 2016) was used for prediction of protein-drug interactions. It provides a comprehensive platform for chemical-protein interactions. The interactions include direct or physical and indirect or functional associations that stem from computational prediction and from interactions pooled from other (primary) databases. The information on virulence factor predictors was derived based on the interaction sequence of E. faecalis, F. nucleatum and P. micros and specific proteins. [12]

Virulence Prediction

VICMpred [13] and VirulentPred [14] pipelines were used to identify virulent factors targeted by APAP and IB among E. faecalis, F. nucleatum and P. micros. The VICMpred server used SVM based method having patterns, aminoacid and dipeptide composition of bacterial protein sequences and overall accuracy of this server was 70.75%. VICMpred grouped proteins into four major classes, namely, proteins involved in cellular process, metabolism, information storage, and virulence. VirulentPred is a bi-layer cascade Support Vector Machine (SVM) based technique for predicting bacterial virulent proteins. The VirulentPred tool categorised the virulent factors into two groups based on the amino acid composition as virulent and avirulent.. The overall accuracy of VirulentPred was 86%. The FASTA format of the proteins retrieved from the NCBI database were used as an input to run the algorithm. [15]

Subcellular localization prediction of the virulent proteins

Computational prediction of protein subcellular localisation assists in the development of novel therapeutic targets or the validation of an antibacterial medication that targets the virulent protein. Cell surface proteins are of great interest because they can be used as vaccine targets. PSORTb V3.0.3 is an algorithm that assigns a protein a localization site based on its amino acid sequence. [16]

B-cell epitopes prediction in the virulent proteins

BepiPred-2.0 is a web server that predicts B-cell epitopes from a protein sequence using a Random Forest algorithm based on epitope and non-epitope amino acids from crystal structures. Residues with scores larger than the cutoff (>0.5) are anticipated to represent epitopes and are highlighted in yellow on the graph. The number of epitopes or antibody binding sites in the pathogenic proteins was also determined. [17,18]

3. Results

Protein-drug interactions in E. faecalis

Proteins interacting with APAP were primarily related to metabolism, followed by cellular processes and information and storage. The scores from VirulentPred marked alkyl hydroperoxide reductase subunit C as virulent proteins (Table 1; Figure 1).

On the other hand, IB was found to react with proteins associated with metabolism followed by cellular processes. All the proteins analysed were avirulent (Table 2; Figure 1).

Protein-drug interactions in F. nucleatum

Proteins interacting with APAP were primarily related to metabolism, followed by virulence factors and cellular processes. GntR family transcriptional regulator, 4-methyl-5(B-hydroxyethyl)-thiazole monophosphate biosynthesis enzyme, glucosamine--fructose-6- phosphate aminotransferase, aminotransferase class-I and 4-amino-4-deoxychorismate lyase were found to be virulent proteins as assessed by VirulentPred scores (Table 1; Figure 1).

Proteins interacting with IB belonged to the class of cellular processes and metabolism. 3-oxoacyl-ACP reductase, long-chain-fatty-acid--CoA ligase, multidrug resistance protein 2 and gluconate 5-dehydrogenase were found to be virulent proteins as assessed by VirulentPred scores (Table 2; Figure 1).

Protein-drug interactions in P. micros

Proteins interacting with APAP were primarily cellular processes followed by metabolism. The scores from VirulentPred marked Creatinase, GroES-like protein, Transcriptional regulator, GntR family, Superoxide dismutase and Glutathione peroxidase as virulent proteins (Table 1; Figure 1).

Proteins interacting with IB belonged to the class of cellular processes and metabolism. Transporter, major facilitator family protein, was marked to be a virulent protein as assessed by VirulentPred scores (Table 2; Figure 1).

Subcellular localization of the virulent proteins

In total, seventeen proteins were marked as virulent based on the scores from VirulentPred. The prediction of the subcellular localization of fifteen virulent proteins revealed scores corresponding to cytoplasmic and cytoplasmic membrane localization of the virulent proteins. The subcellular localization of two virulent proteins could not be found and hence, were marked as unknown. Furthermore, the numbers of epitopes or antibody binding sites in all the seventeen virulent proteins were also determined (Table 3; Figure 2,3).

4. Discussion

In silico validation is required when choosing a chemical or drug to be researched in vitro or in vivo. Apart from lowering the cost of conducting trials, it also provides information on the particular mechanism or pathway that may be targeted by these virulent proteins, perhaps shortening the process and making it more focused. In this context, the present study was designed to discover the possible interactions between the endodontic pathogens with APAP and IB.

According to a study conducted in 2019, the incidence of E. faecalis might reach as high as 70% in cases of secondary root canal infections. [19] Bronzato et al conducted a study which aimed to investigate the microbial profile of periapical lesions associated with teeth after root canal treatment and retreatment. The study results showed the polymicrobial nature of the periapical lesions. P. micra was the most frequently detected microbe in all groups, followed by E. faecalis, F. nucleatum, and P. endodontalis. [20] In infections of such polymicrobial nature, maximizing infection eradication is important to increase the chances of success.

IB, one of the commonest used NSAID in the treatment of reversible or irreversible pulpitis is notable for its anti-inflammatory activity, whilst APAP works by exerting an analgesic effect, thus reducing pain in case of such infections. [21] In addition to anti-inflammatory and analgesic properties, antibacterial activity has also been demonstrated in various in vitro studies. [1,6,8] As a result, non-antibiotic drugs can be used to treat microbial infections. The current work adds to our understanding of the potential antibacterial activity of APAP and IB by triggering molecular

mechanisms that contribute to their bactericidal or bacteriostatic activity.

A study conducted by Ferrer-Luque et al on the antibiofilm activity of diclofenac and antibiotic solutions in endodontic therapy showed that diclofenac solutions at 5% and 2.5% had greater antimicrobial effects than triple antibiotic solution and double antibiotic solution and may be considered a valid alternative for controlling the infection of teeth with apical periodontitis. [22] İsmail ÖZTÜRK et al studied the antibacterial properties of NSAIDs on Staphylococcus aureus and highlighted that all NSAIDs were active against S. aureus strains (MIC values ranging from 195 µg/mL to 6250 µg/ mL). The authors also highlighted that NSAIDs increased the antibiotic susceptibility of the strains. [7] An in vitro study investigated the potential antibacterial effects of ibuprofen and acetaminophen on pathogenic bacteria and reported that Staphylococcus aureus and Paracoccus yeei were susceptible to lower concentrations of ibuprofen and acetaminophen (MIC=1.25 mg/ml), while two strains of Enterobacter exhibited resistance to these agents. [8] As a result, these drugs must be rigorously tested against a range of pathogens in order to find potential mechanisms of sensitivity or resistance. A study was conducted in 2018 on the anti-candidal activity of selected analgesics. The minimal inhibitory concentration as well as fractional inhibitory concentration of selected analgesics and triazole on eighteen Candida species isolates were determined by the standard microdilution method according to EUCAST. Among five tested compounds, only ibuprofen showed an antifungal effect against all tested isolates. [23]

In a review, Yin et al. emphasized the molecular mechanism behind the antibacterial effectiveness of NSAIDs against E. coli. NSAIDs inhibited the beta subunit of Escherichia coli DNA polymerase III, a crucial interaction hub that serves as a mobile tether on DNA for numerous key partner proteins involved in DNA replication and repair. The NSAIDs were bound to the sliding clamp at a common binding region essential for partner binding, according to crystal structures. An in vitro DNA replication experiment showed that the clamp loader and/or the replicative polymerase alpha subunit were inhibited from interacting with the sliding clamp. As a result, NSAIDs are excellent lead scaffolds for new antibacterial drugs that target the sliding mechanism. [24]

Most in vitro research to date have established the antibacterial activity of APAP and IB, but only a few have elucidated the molecular pathways behind the antimicrobial activity. [25,26,27] The current study is the first of its type, revealing many key protein interactions between two routinely used medications, APAP and IB, against endodontic microbes. Multiple virulence factors, as well as proteins involved in cellular functions and metabolism, were discovered to be targeted by APAP and IB. However, the processes that cause bacteria susceptibility must be addressed through additional in vitro research in order to justify their inclusion in the list of bactericidal drugs.

A few limitations of the study are as follows: 1) The interactions between drugs and proteins may merely be physical, with or without functional implications; 2) Certain host proteins may have similarity with microbial proteins, in this case, the targets should be carefully chosen to have minimal amount of negative impact on the host.; and 3) In silico approaches may not accurately reflect protein interactions that occur in vivo, as other proteins in the proximity may interfere with the validated functional route. Future research in this area may assist in establishing the synergistic and antagonistic effects of these drugs when used in conjunction with standard antibiotics. Because the toxicity and efficacy of APAP and IB have previously been demonstrated, utilizing these drugs against pathogenic bacteria is not a concern. In vitro and in vivo research should be used to determine the drug dosage, minimum inhibitory concentration, and minimum bactericidal concentration.

5. Conclusion

This study identified molecular targets of APAP and IB on E. faecalis, F. nucleatum and P. micros which must be confirmed under physiological circumstances to understand the essential pathways triggered by these drugs. In the antibiotic-resistant era, in silico analysis may assist in the target specific localization of antimicrobial agents, potentially opening up new techniques to find antimicrobial agents and treat pathogenic organisms.

Conflict of Interest
The authors declare no conflict of interest.

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Table 1: Protein repertoires of endodontic Pathogens interacting with acetaminophen

Organism	**Identifier**	**Protein**	**VICMPred Functional Class**	**VirulentPred**	**VirulentPred Score**
Enterococcus Faecalis	EF0117	GntR family transcriptional regulator	Cellular Process	Avirulent	-1.048

	EF2994	class V aminotransferase	Cellular Process	Avirulent	-1.011
	EF2426	GntR family transcriptional regulator	Metabolism Molecule	Avirulent	-1.046
	EF1793	branched-chain amino acid aminotransferase	Metabolism Molecule	Avirulent	-1.046
	EF2739	alkyl hydroperoxide reductase subunit C	Cellular Process	**Virulent**	0.2244
	EF1597	catalase/peroxidase	Cellular Process	Avirulent	-1.024
	EF0109	ThiJ/PfpI family protein	Information and Storage	Avirulent	-1.053

	EF1951	phosphosugar-binding protein	Metabolism Molecule	Avirulent	-0.986
	EF2151	glucosamine—fructose-6-phosphate aminotransferase	Metabolism Molecule	Avirulent	-0.575
	EF2555	thymidine kinase	Metabolism Molecule	Avirulent	-1.027
Fusobacterium Nucleatum	FN1462	GntR family transcriptional regulator	Cellular Process	**Virulent**	1.0299
	FN0452	glucosamine--fructose-6-phosphate aminotransferase	Metabolism Molecule	Avirulent	-0.966

	FN1876	4-methyl-5(B-hydroxyethyl)-thiazole monophosphate biosynthesis enzyme	Metabolism Molecule	**Virulent**	1.0272
	FN1983	alkyl hydroperoxide reductase	Metabolism Molecule	Avirulent	-1.078
	FN1085	4-methyl-5(B-hydroxyethyl)-thiazole monophosphate biosynthesis enzyme	Virulence Factors	Avirulent	-0.763
	FN0628	glucosamine--fructose-6-phosphate aminotransferase	Metabolism Molecule	**Virulent**	1.0356

	FN0334	aspartate/aromatic aminotransferase	Virulence Factors	Avirulent	-1.007
	FN1416	aminotransferase class-I	Metabolism Molecule	**Virulent**	0.2051
	FN1418	aminotransferase class-I	Metabolism Molecule	**Virulent**	0.2244
	FN1729	4-amino-4-deoxychorismate lyase	Metabolism Molecule	**Virulent**	0.8751
Peptostreptococcus Micros	PEPMIC_01705	Creatinase	Cellular Process	**Virulent**	0.7748
	PEPMIC_01672	Thymidine kinase	Cellular Process	Avirulent	-1.003

	PEPMIC_01365	GroES-like protein	Cellular Process	**Virulent**	0.4303
	PEPMIC_00941	L-glutamine--D-fructose-6-phosphate amidotransferase	Metabolism Molecule	Avirulent	-1.047
	PEPMIC_01145	Acyl-CoA dehydrogenase, C-terminal domain protein	Metabolism Molecule	Avirulent	-1.024
	PEPMIC_01143	DJ-1 family protein	Cellular Process	Avirulent	-0.969
	PEPMIC_00213	Transcriptional regulator, GntR family	Cellular Process	**Virulent**	0.5423

	PEPMIC_01380	Peroxiredoxin	Cellular Process	Avirulent	-0.420
	PEPMIC_01189	Superoxide dismutase	Cellular Process	**Virulent**	0.9914
	PEPMIC_00450	Glutathione peroxidase	Metabolism Molecule	**Virulent**	0.0540

Table 2: Protein repertoires of endodontic pathogens interacting with Ibuprofen

Organism	**Identifier**	**Protein**	**VICMPred Functional Class**	**VirulentPred**	**VirulentPred Score**
Enterococcus Faecalis	EF0629	aldo/keto reductase family oxidoreductase	Metabolism Molecule	Avirulent	-0.964
	EF1773	3-ketoacyl-ACP reductase	Metabolism Molecule	Avirulent	-0.149

	EF1138	aldo/keto reductase family oxidoreductase	Cellular Process	Avirulent	-1.008
	EF2382	glucose 1-dehydrogenase	Metabolism Molecule	Avirulent	-1.044
	EF0452	AMP-binding family protein	Metabolism Molecule	Avirulent	-0.969
Fusobacterium Nucleatum	FN0216	3-oxoacyl-ACP reductase	Cellular Process	**Virulent**	0.9715
	FN1122	long-chain-fatty-acid--CoA ligase	Metabolism Molecule	**Virulent**	1.0331

	FN1497	multidrug resistance protein 2	Cellular Process	**Virulent**	0.9486
	FN0867	long-chain-fatty-acid--CoA ligase	Metabolism Molecule	Avirulent	-1.050
	FN0107	Sodium/proline symporter	Metabolism Molecule	Avirulent	-1.015
	FN1687	gluconate 5-dehydrogenase	Cellular Process	**Virulent**	0.9756
Peptostreptococcus Micros	PEPMIC_01287	Uridine phosphorylase	Cellular Process	Avirulent	-1.011
	PEPMIC_00554	Transporter, major facilitator family protein	Metabolism Molecule	**Virulent**	0.8063

Table 3: Subcellular localization of virulent protein as predicted by PSORTb V3.0.3

Identifier	**Virulent Protein**	**Subcellular Localisation of Protein**	**Score**
EF2739	alkyl hydroperoxide reductase subunit C	Cytoplasmic	9.97
FN1462	ntR family transcriptional regulator	Cytoplasmic	9.97
FN1876	4-methyl-5(B-hydroxyethyl)-thiazole monophosphate biosynthesis enzyme	Cytoplasmic	8.96
FN0628	glucosamine--fructose-6-phosphate aminotransferase	Cytoplasmic	9.97
FN1416/FN1418	aminotransferase class-I	Cytoplasmic	9.97
FN1729	4-amino-4-deoxychorismate lyase	Cytoplasmic	9.94

PEPMIC_01705	Creatinase	Cytoplasmic	7.50
PEPMIC_01365	GroES-like protein	Unknown	-
PEPMIC_00213	Transcriptional regulator, GntR family	Cytoplasmic	9.97
PEPMIC_01189	Superoxide dismutase	Extracellular	9.60
PEPMIC_00450	Glutathione peroxidase	Unknown	-
FN0216	3-oxoacyl-ACP reductase	Cytoplasmic	9.26
FN1122	long-chain-fatty-acid--CoA ligase	Cytoplasmic Membrane	7.88
FN1497	multidrug resistance protein 2	Cytoplasmic Membrane	10
FN1687	gluconate 5-dehydrogenase	Cytoplasmic	9.97

PEPMIC_00554	Transporter, major facilitator family protein	Cytoplasmic Membrane	10

Figure 1:
A) Protein repertoires of endodontic Pathogens interacting with acetaminophen
B) Protein repertoires of endodontic Pathogens interacting with ibuprofen

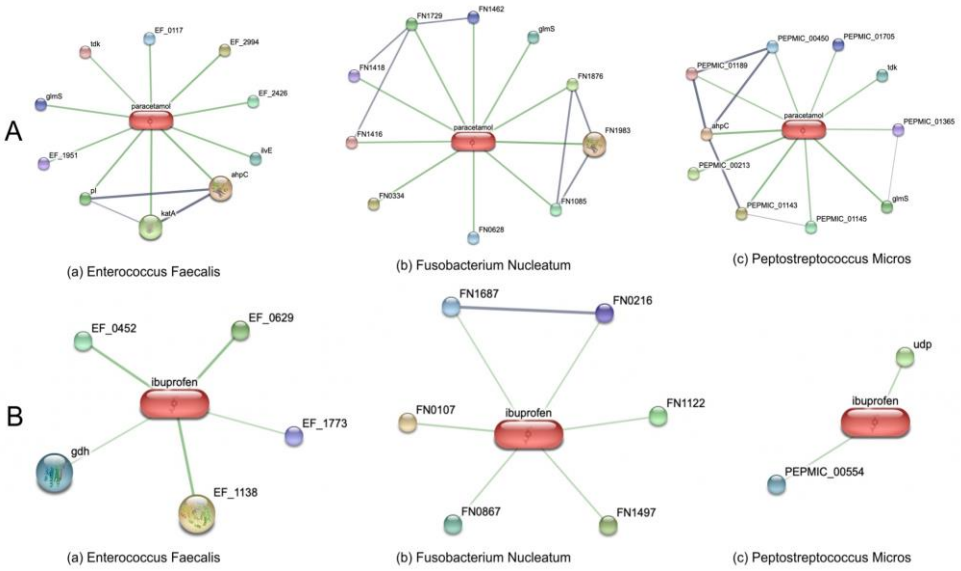
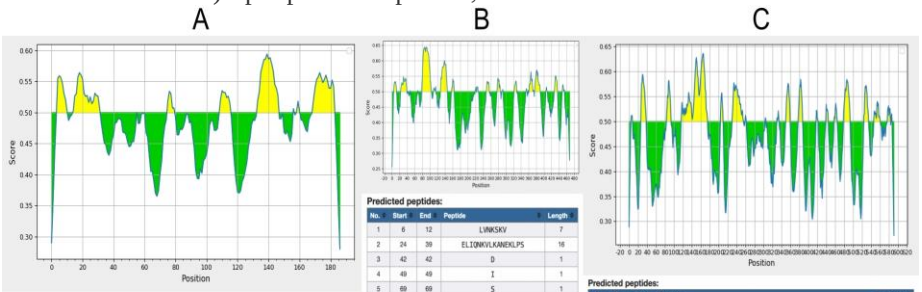


Figure 2: Epitopes in the virulent proteins of endodontic pathogens targeted by acetaminophen
A) Epitopes in the protein, alkyl hydroperoxide reductase subunit C of E. faecalis
B) Epitopes in the protein, GntR family transcriptional regulator of F. nucleatum
C) Epitopes in the protein, creatinase of P. micros

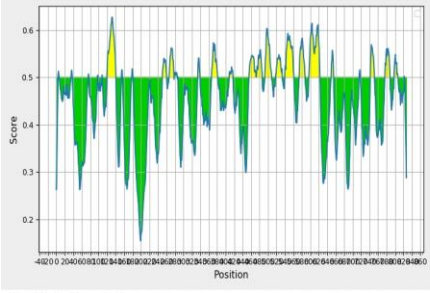


Predicted peptides:

No.	Start	End	Peptide	Length
1	5	11	NQKLFDF	7
2	15	32	AYHDGEFTRVSTEDILGK	18
3	76	80	HYVHK	5
4	110	116	VLDEASG	7
5	134	147	YEINDMGIGRNAEE	14
6	157	157	F	1
7	159	161	AEH	3
8	170	183	WQPGEETIAPSLDL	14

Figure 3: Epitopes in the virulent proteins of endodontic pathogens targeted by ibuprofen
A) Epitopes in the protein, long-chain-fatty-acid--CoA ligase of F. nucleatum
B) Epitopes in the protein, Transporter, major facilitator family protein of P. micros

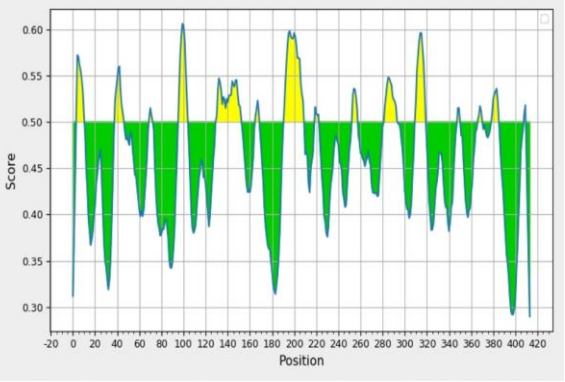
A



Predicted peptides:

No.	Start	End	Peptide	Length
1	5	7	TDK	3
2	36	38	KIK	3
3	77	77	V	1
4	98	99	VE	2
5	109	109	S	1
6	121	141	NLSEIKIDENSSENLVINSPE	21
7	155	156	TG	2
8	180	181	MY	2
9	252	261	DTINSKAITR	10
10	267	278	AKKVNSLSFSKL	12
11	280	286	FKKVSEG	7
12	288	288	G	1
13	335	340	NNIVFD	6
14	368	382	KGYYKNPEATVEIID	15
15	410	418	IVLSNGKNI	9
16	456	487	KVKEEKVDNIYENLKWEVVDKYNQKTPDYKKI	32
17	494	509	NEDFPKTKIGKIKRFM	16
18	519	534	EKQERKPEPDFEEYNK	16
19	541	562	DIKGKDVYFDSHIEIDLGMDSL	22
20	575	588	YGIKEENLISKYPT	14
21	598	623	GNRNQEKIGNLDWKEIVNKDTTAKLP	26
22	651	655	KEKIE	5
23	717	719	KDI	3
24	743	752	LRTRDGKMNK	10
25	777	789	YDLFPAGKKFPRP	13
26	799	808	KIKVEKLTYD	10
27	823	823	E	1

B



Predicted peptides:

No.	Start	End	Peptide	Length
1	4	11	LKESRKQM	8
2	39	47	KTESALNFG	9
3	70	73	KYNK	4
4	97	104	SNTETNLI	8
5	130	153	KLVCEEHIQKVKSFEQMASSGVYI	24
6	166	169	LDLI	4
7	192	209	DFIKTEVIENNEEQKVLK	18
8	220	223	DKKA	4
9	253	258	IKISDY	6
10	282	294	KDIKAPLEFSFKC	13
11	311	320	FKFSIFSYFI	10
12	349	351	PLE	3
13	367	371	LMPLG	5
14	380	386	DKFKSGY	7
15	409	410	LK	2