SUMMARY & GENERAL CONCLUSIONS

This research project aimed to contribute to our understanding of microbial-host interactions using quorum sensing peptides (QSPs). More specifically, the effects of QSPs on brain functioning were investigated. In **Chapter I,** the clear link between the gut microbiome and the brain and its effects on behaviour and brain functioning is discussed. In addition, concepts as 'the microbiome' and 'quorum sensing peptides' are explained. Peptides are able to cross the blood-brain barrier and exert diverse effects in the brain. Examples of both peptides with therapeutic and toxic properties are discussed, indicating the potential of quorum sensing peptides to affect the brain and be mediators of the gut-brain axis.

In the **second chapter**, the development of a microbiome-disease association database called: 'Disbiome' is discussed. This database contains information concerning microbiome differences between a patient population and a (healthy) control population. Every microbial species (bacterium, virus, fungus…) which is differentially abundant between a patient population and a control population in a particular disease is presented as an 'experiment' in the database. Information concerning the investigated sample type, sample location, used detection method, methodological details, investigated population and publication are all linked to this experiment. Both the diseases as microbial species included in the database are classified using standardized systems, respectively the MedDRA and the NCBI/SILVA taxonomies. The quality of manuscripts reporting these microbiome-disease associations is also assessed using a questionnaire of 16 questions each evaluating different aspects of the manuscript. Based on this questionnaire, it is observed that reporting of some parameters change over time. Indicating that reporting and/or the design (*e.g.* other detection methods…) of this type of research evolved over time. To date, the database contains over 1000 publications and forms an extensive dataset to study microbiome-disease associations.

In **chapter III**, a screening of 85 QSPs is performed using high-throughput *in vitro* screening experiments on different brain cell types and using different biological outcomes such as changes in morphology, cytokine production and cytotoxicity. Based on these initial screening experiments, 25 peptides were found to exert effects on either one or multiple brain cell lines. However, the majority of the observed effects were false positives as these results could not be replicated in confirmation experiments using different peptide concentrations and a higher number of replicates. Two peptides showed promising results for further investigation. PapRIV showed a non-significant increase of both IL-6 and TNFα, two pro-inflammatory cytokines, after BV-2 microglia treatment; indicating that this

peptide could activate microglia cells and play a causative role in neuro-inflammation and related psychiatric- and neurodegenerative disorders. Orf4 showed induction of differentiation in SH-SY5Y neuroblastoma cells, indicating that this peptide could have a protective role in the development of neuroblastoma. Based on these results, PapRIV was chosen for further in depth investigation in this thesis seen the importance of neuro-inflammation in different neurological disorders.

In **chapter IV**, the pharmacokinetic profile of PapRIV is investigated using both *in vitro* and *in vivo* models. The peptide was able to cross the *in vitro* Caco-2 model, a model of the gastro-intestinal tract, with a P_{app} of 1.37 ± 0.21 $x10^{-9}$ cm/s. Once entered the blood stream, the peptide reaches the blood-brain barrier where a very high brain influx occurs with a K_i of 6.95 µl/(g x min) and a brain distribution volume of 35.70 µL/g as indicated with an *in vivo* mouse model. However, in the circulation and tissues, also a strong metabolization occurs with observed half-lives of 20 min, 25 min, 90 min, 285 min and 523 min in respectively, kidney, serum, faeces, liver and brain tissue while no metabolization was observed in colon tissue. Different metabolites were formed with DLPFEH being the main metabolite formed in all tissues. This data indicates that PapRIV, which is produced in the gut, is able to cross the gastro-intestinal tract and blood-brain barrier to reach the brain and exert it's possible microglia activating effects.

In the following **chapter five**, endogenous presence of PapRIV is demonstrated in mouse plasma. Using a highly specific UHPLC-MS system in multiple-reaction monitoring mode, the peptide was detected in 4 out of 66 plasma samples with detected concentrations ranging from 1.7 to 19.2 nM. Using more high-resolution (but less sensitive) MS systems, presence of the peptide could be confirmed in two of these samples. For the first time, the *in vivo* presence of a QSP is demonstrated. Based on BLAST results, it is concluded that the peptide's sequence is not present in both the mouse and human proteome; indicating that the detected peptide does not originate from proteolytic cleavage of endogenous proteins and that the detected peptide is thus from bacterial origin.

The pro-inflammatory microglia activating properties of PapRIV is further investigated in **chapter six**. Treatment of BV-2 microglia cells confirmed the microglia activating properties of this QSP as a significant increase of IL-6, TNFα and reactive oxygen species was observed. These effects were accompanied by an increased fraction of ameboid cells, another marker for microglial activation. The observed effects were mediated by activation of the canonical NF-κB pathway, as an increased NF-κB nuclear translocation and a decreased IκBα expression was seen in the cells. By using an alanine scan of the peptide sequence, critical amino-acids of the peptide could be identified. When replacing aspartic acid or proline at the second and fourth position with an alanine residue, the corresponding peptides were not able anymore to exert activating effects; indicating that these two residues are

necessary for the peptide's actions. PapRIV can also play an indirect role in neurodegenerative disorders as treatment of SH-SY5Y neuroblastoma cells with BV-2 conditioned medium (medium of PapRIV treated BV-2 cells) resulted in cytotoxicity while the peptide showed no direct toxic effects towards these cells. Investigation of the metabolites, identified in chapter four, showed that DLPFEH, the main metabolite of PapRIV, remains active towards BV-2 microglia cells. By co-incubating the peptide with different inhibitors of membrane-associated receptors, we tried to identify the target receptor of PapRIV. Based on the results, we can conclude that PapRIV does not act on the P2X1-, P2X3-, P2X4-, LPA and mineralocorticoid receptors. However, no clear conclusion on the NMDA-, P2X7-, glucocorticoid- and amylin receptors could be made as inhibition of these receptors resulted in IL-6 levels which are even lower compared to placebo treated cells. Indicating that baseline activity of these receptors, which cause IL-6 expression, is also present in placebo treated cells. It is possible that PapRIV act as an agonist on these receptors, but we cannot conclude on this.

The results of PapRIV are however only seen in the murine BV-2 microglia cell-line. The peptide showed no effects in both murine and human induced pluripotent stem cells which are differentiated towards a microglia-like phenotype. Also *in vivo*, no effects were seen after intracranial injection directly in the brain cortex; questioning the *in vivo* relevance of this peptide.

Finally, in **chapter seven**, the effects of peptides on epigenetic machineries are discussed. Different peptides from different sources such as food (*e.g.* lunasin), endogenous (*e.g.* short cryptic peptides), the environment (*e.g.* acyldepsipeptides) and synthetic developed peptides modulate different epigenetic mechanisms. Effects observed are mainly the inhibition of: histone (de)acetylation, histone (de)methylation and DNA methylation. Peptides can also stimulate or suppress Dicer mediated miRNA maturation resulting in higher or lower expression of certain miRNAs. Seen these effects, this can have some implications in further peptide therapeutic development. To date, over 60 peptide therapeutics are approved and on the market, with its epigenetic (side)effects largely unknown. Discovery of epigenetic effects of these products can open new avenues for the development of new treatments via drug repurposing. In addition, harming (long-term) epigenetic side effects, which can give rise to malignant transformations, can be detected.

To conclude, the relevance of this research project in a broader international context is discussed and some suggestions are made about how this work could form the basis for further investigations and development of applications.