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The influence of *Akkermansia muciniphila* on intestinal wound healing

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Summary

Despite technological developments and improvements in the perioperative management of colorectal surgery, (e.g. technology advances, prehabilitation programs starting prior to surgery and enhanced recovery programs after surgery), colorectal anastomotic leakage continues to be a devastating surgical complication with a prevalence of up to 19% and is associated with a significant increase of mortality.

To date, the exact mechanisms of anastomotic leakage development are still not completely deciphered although it is well- recognised that numerous interacting factors are involved and preventing measures have been proposed to reduce their impact when possible.

Recent literature also supports the idea that the microbiota has a strong influence on the healing of an intestinal anastomosis. Understanding the mechanisms by which the bacterial community of the gut influences intestinal healing could open the door for new preventive and therapeutic approaches.

Animal models are a useful tool to study and decode the mechanisms leading to anastomotic leakage, however the existing murine anastomotic leakage models have major limitations and are not suited to assess the consequences of local microbiota manipulation.

Therefore, we developed and described a novel intestinal wound healing model called the "***Colonoscopic Leakage Model***" in which the colon is locally perforated to mimic colonic leakage. This innovative model is less invasive than other currently used models allowing us to study the underlying molecular mechanisms of interaction between the local microenvironment and the intestinal wound healing process with less confounding factors. Furthermore, because it is reproducible, fast, and easy to perform, it

represents a potential high-throughput model for the validation of different pharmacological and/or therapeutic tools.

Using this model, we found that the administration of ***Akkermansia muciniphila,*** a next-generation beneficial bacterium that affects the gut barrier function and the local immunity, could modulate the intestinal environment, and accelerate healing, thereby reducing the risk for anastomotic leakage and enhancing post-surgery recovery. Seven days after colonic perforation, *A. muciniphila* treated mice had **significantly lower mortality, and severity of peritonitis**. This effect was associated with significant improvements of wound histological healing scores, higher production of IL22 without affecting the mucus layer thickness or genes involved in cell renewal, proliferation, or differentiation.

All these effects were abolished in mice deficient for MYD88, a cytosolic adapter protein that plays a central role in the innate and adaptive immune response, showing the involvement of this protein in this process.

Finally, in a proof-of- concept pilot study, healthy humans were exposed to *A. muciniphila* for 2 hours and colonic biopsies were taken before and after colonic instillation for transcriptomic analysis. Results showed that human subjects exposed to *A. muciniphila* exhibited significant changes in the expression of genes in their colonic mucosa, showing the capacity of this bacterium to influence its host physiology.

Together, these experiments suggest that *A. muciniphila* improves wound healing after transmural colonic wall defect through **mechanisms possibly involving IL22 signaling and requiring MyD88 in the intestinal cells**. In healthy humans, colonic administration of *A. muciniphila* is **well-tolerated and changes the expression of gene**s involved in the immune pathways.

Preventive measures, like the implementation of mechanical bowel preparation prior to colorectal surgery, have not translated into a decreasing rate of the anastomotic fistula rate. The need for appropriate interventions remains high. It is therefore crucial to continue and expand our knowledge on anastomotic leakages in order to improve the clinical routine. We believe that in depth-microbiota based analysis are an important aspect of this research. Based on our results, we could imagine developing a **completely innovative bowel preparation concept** through which the microbial community would be modified so that beneficial bacteria would be administered or stimulated while eliminating bacteria detrimental to the healing process.

List of abbreviations and acronyms

Akk	Akkermansia muciniphila
AL	Anastomosis leakage
Ang1	Angiopoietin 1
Angg1	Angiogenin 1
AMPs	Antimicrobial peptides
AI	Artificial intelligence
CVD	Cardiovascular diseases
COL1A	Collagen 1A
CT	Control
CCND1	Cyclin D1
DAMPs	Damage-associated molecular patterns
ERAS	Enhanced recovery after surgery
ERK	Extracellular signal-regulated kinase
FT	Fast-Track
FOXP3	Forkhead box P3, also known as scurfin
FPR	Formyl peptide receptor
GPR	G protein-coupled receptors
GLP	Glucagon-like peptide
GI	Gastro-intestinal
IEC-MyD88KO	Inducible intestinal epithelial cell-specific MyD88 Knock-out
IEC	Intestinal epithelial cells
ICAM2	Intracellular adhesion molecule2
i.p.	Intraperitoneally
KO	Knock-out
LOS	Length of stay

LPS	Lipopolysaccharides
Lyz1	Lysozyme 1
MARCO	Macrophage receptor with collagenous structure
MMP2	Matrix metalloproteinase 2
MBP	Mechanical bowel preparation
MyD88	Myeloid differentiation primary response 88
NIRF	Near-infrared fluorescence
NGN3	Neurogenin-3
NeuroD1	Neuronal Differentiation 1
NOX2	Neutrophilic NADPH oxidase
NAFLD	Non-alcoholic liver diseases
OA	Oral antibiotics
PAMPs	Pathogen–associated molecular patterns
PPAR	Peroxisome proliferator-activated receptor
PRRs	Patter recognition receptors
Pla2	Phospholipase A2
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Reg3γ	Regenerating islet derived-3 gamma
SOPF	Specific and opportunistic pathogen free
TLRs	Toll-like receptors
TRAIL	TNF-related apoptosis-inducing ligand
T2D	Type-2 diabetes
WT	Wild Type

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INTRODUCTION

1 The anastomotic leakage

1.1 Anastomosis development throughout history

An anastomosis is a surgical procedure performed to join two formerly distant structures in the human anatomy. Already the early surgeons, the so-called wound doctors, tried to fix the different gut defects by suturing or *anastomosing* the intestine. Various surgical methods can be traced back to the third millennium BC [1].

The first more precise descriptions of how to perform intestinal sutures were provided by the doctors Roger, Jamerius and Guglielmo da Saliceto in Italy in the 14th century. They recommended splinting an intestinal suture with an endoluminally inserted prosthesis. This consisted of animal dried trachea, which was supposed to stabilise the intestinal suture and was not fixed to the intestinal wall so that it could later be excreted via naturalis [8].

Until the late 19th century, the indications for anastomosis were only in case of traumatic intestinal injuries and gangrenous parts of the intestine, mostly because of incarcerated hernias. Small intestine wounds were considered in principle fatal, while stomach or colonic injuries were treated surgically [2].

Various factors contributed significantly to the further development of intestinal anastomosis. In particular, the discovery of procedures to create aseptic conditions by Lord Joseph Lister in 1867 is certainly the most important basis for successful intestinal anastomosis [3].

The innovation of suture materials, the development of anaesthesia, the discovery of the necessity of serosa apposition, as well as the need to invert the ends of the intestine described in 1826 by Antoine Lembert, made the current routine of anastomosis possible. These advances allowed to expand

the range of indications for anastomosis, further gaining valuable clinical-surgical experience [4].

1.2 Anastomotic Leakage

Anastomotic leakage occurs when a surgical anastomosis fails and is defined as a breach in a surgical join between two hollow viscera, with or without active leak of luminal content (although there are some minor derivations) [5] (Figure 1). It remains a major and potentially life-threatening complication following colorectal surgery.

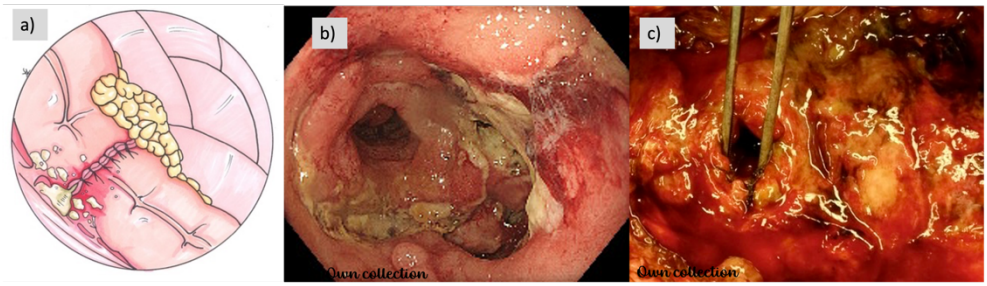


Figure 1: Anastomotic Leakage

a) schematic overview
b) endoscopic view
c) surgical view

Despite numerous technical surgical advancements and the development of perioperative enhanced recovery methods in the last decades [6, 7], the percentage of colonic anastomotic leakages did not improve significantly. Literature shows that the prevalence of anastomotic leakage ranges from 1 to 19 %, depending on the anatomical site, and that, in addition to the

economic implications, this has direct impact on survival, quality of life, and cancer recurrence [8, 9]. The mortality rates after the appearance of an anastomotic leakage vary in the literature typically from 15 to 33% [10-12].

1.3 Factors involved in the occurrence of an anastomotic leakage

[Adapted from the review Bachmann et al., published in GUT 2017]

The factors influencing the anastomotic healing are already well described and studied and different preventive strategies against anastomotic leakages have been proposed [13-15]. Kirchhoff for example, classified these factors into two categories: factors that can be influenced (e.g. obesity, nutritional status, preoperative bowel cleaning, experience of the surgeon) and predisposing or risk factors that cannot be controlled (e.g., age and gender, previous history of radiotherapy) (Figure 2) [16].

Furthermore, a distinction is made between an early form of anastomotic insufficiency (up to 3 days postoperatively), which is usually caused by a technical problem, and a late form of anastomotic insufficiency (from the 3rd postoperative day on) which appear because of a delay in the healing caused by the different factors.

Strikingly, many of these risk factors have also been shown to influence the composition of the microbiota [17], while at the same time they can themselves be deeply impacted by the microbiota, suggesting thus the probability that the gut microbiota has a role in intestinal wound healing [8].

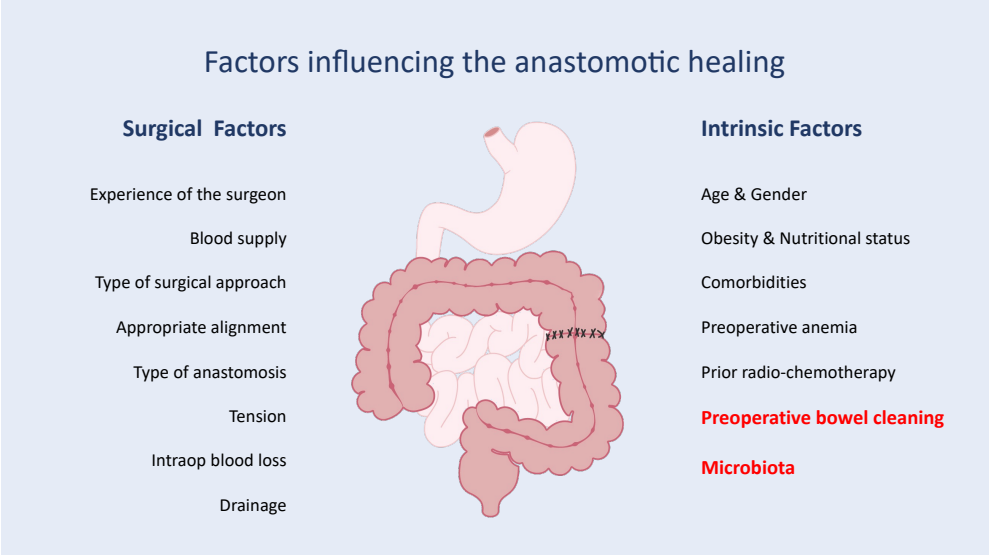


Figure 2: Factors influencing the anastomotic healing [8].

Incrementally papers showed recently that the local microenvironment, and implicit the bowel preparation, play an essential role in the wound healing

1.4 Current clinical practice in colorectal surgery

The management of the colorectal surgery has progressed significantly over the past century [18]. Following the introduction of laparoscopic abdominal surgery, the next stage in the evolution of this specialization began in the 1990s with the first laparoscopic colonic resection. Despite concerns expressed in early stages, numerous prospective studies as well as meta-analyses showed the oncological noninferiority of this approach compared with the open approach [19], while having, in addition, all the advantages of a minimal invasive approach.

In 1997, another revolutionary idea improved the peri- and post operative management in colorectal surgery dramatically, with the Danish surgeon Henrik Kehlet introducing the concept of enhanced rehabilitation after surgery (ERAS), or Fast-Track (FT), in colorectal surgery, aiming at minimising the impact of surgical stress on the physiological homeostasis of the patient [20, 21]. By using this protocol, it was possible to significantly decrease the length of hospital stay (LOS), and to reduce postoperative complications. Unfortunately, this protocol did not lead to a decrease of the anastomotic leakage rate.

Although the programme originally consisted of only four points (minimally invasive surgery, multimodal analgesia based on thoracic epidural, early enteral nutrition, and early mobilisation), other measures were added further on to optimise the preservation of the patient's bowel function and mobility in order to bring the patient back to the pre-operative level as soon as possible after major surgery.

In the last decade the concept of *prehabilitation* was introduced [22, 23]. This is a multidisciplinary preoperative program aiming to improve physical condition, nutritional status, and preoperative anxiety with an optimal duration of the program between 2 and 8 weeks prior to the surgery, depending on the feasibility [24]. The final purpose of the *prehabilitation program* is to reduce postoperative complications.

In addition to the standardization of the different surgical techniques associated with the implementation of histological quality control, another major improvement in the colorectal surgery management is the continuous technical improvement of the equipment with strong digitalization trends and the potential future use of artificial intelligence (AI) combined with virtual and/or augmented reality [25, 26].

In this respect, a good example is the introduction over the last two decades of the robotic-assisted surgery [27] with enhanced three-dimensional (3D) visualisation and magnification to augment the surgeon's depth of perception and clarity of vision combined with the use of articulated instruments which provide enhanced movement possibility, beyond the limits of human wrist operations and eliminates human hand tremors, thus facilitating superior operative dexterity with augmented precision for surgical dissection while preserving stable tissue retraction.

Another important technical advancement used routinely nowadays in colorectal surgery, is the verification of a good vascularization at the anastomotic level using near-infrared fluorescence (NIRF). This is a technique of augmented reality which allows the colorectal surgeon to visualize and assess bowel vascularization [28], when applied in the operating theatre.

However, all these recent advancements and the net amelioration of the postoperative morbidity did not translate into any improvements in the anastomotic leakage rate [29].

2 The intestinal microbiota

2.1 Bacteria in the human body

The human body hosts whole communities of microorganisms in the areas of contact with the outside world. While the contact surface of the skin is about 2 m^2, that of the intestine with all its villi is between 250 and 400 m^2 [30], making it the home of the most densely populated microbial ecosystem that

colonizes the human body, commonly referred to as the intestinal/gut microbiota. This gut microbiota comprises hundreds of trillions of bacteria but also bacteriophagic particles, viruses, fungi and archaea.
The intestinal microbiota represents 95% of all bacteria in the human body [31], weighing approximately 1 to 2 kg.

The distribution of bacteria along the digestive tract is not homogeneous [32] (Figure 3). While the stomach is a very poor environment for bacteria (about 100 bacteria per gram of stomach content) because its acidic pH and the presence of digestive enzymes are unfavourable to bacterial growth, the concentrations of bacteria gradually increase along the digestive tract, reaching a maximum in the colon (about 1000 billion bacteria per gram of intestinal content), where the conditions there are optimal: the temperature is constant (37°C), the environment is not very acidic and rich in water, the transit is slow, and food is abundant [33].

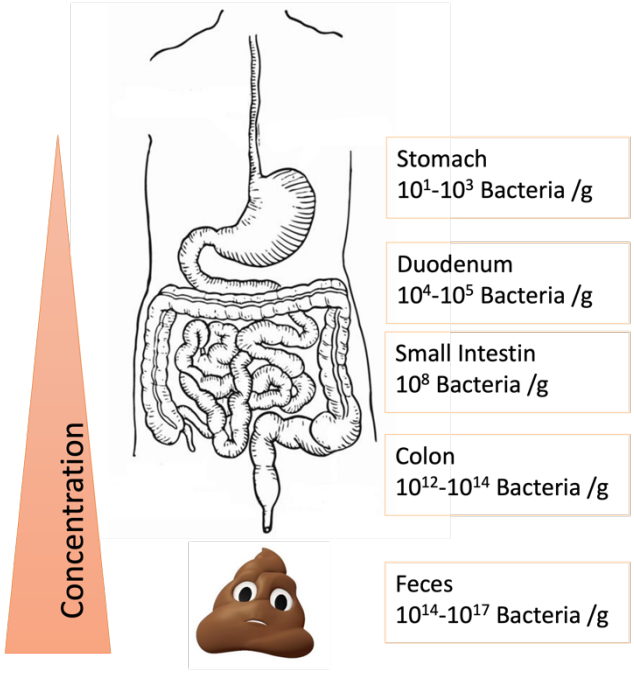


Figure 3: Distribution of the bacteria within the human body

Total abundance of bacteria according to the different body sites [17,34,35]. Bounds for bacteria number in different organs, derived from bacterial concentrations and volume.

Depending on the calculation method (lapse of time since the last defecation and whether or not red blood cells are taken into account [34, 36]) our body harbours about 100 thousands of billions of bacteria, which is about 1 to 10 times more than our human body cells [34].

When considering the amount of genetic information, the microbial genetic information dwarfs the human genome with a genetical capacity that exceeds ours by approximately hundred times, indicating that these microorganisms have metabolic and biological functions that cannot be performed by our human metabolism. This suggests that most of what is going on genetically in our body is in fact microbial and that there is an important cross talk taking place between the host and its microbiota [37].

There are at least 1000 different species of bacteria in our gut microbiota [38]. The composition of the microbiota varies not only within and between tissues [39] but also between individuals and in the same person depending on age, on diet and lifestyle, particularly upon onset of disease and frailty [40, 41].

Although knowledge on the different bacteria that inhabit our gut has grown substantially over the last few years, it is worth noting that recent development of culture-independent methods has revealed even greater diversity within the bacterial communities of the mucosa than were previously identified using traditional culture-based methods [42]. It is estimated that the vast majority of bacterial species in the human microbiota have not yet been cultured because a great number of the unidentified bacteria have complex

and dynamic growth requirements that render them difficult to reproducibly isolate [43].

2.2 Microbes and human health

If we imagine the Earth's history, from its creation with the appearance of life till today, as a calendar month, bacteria would already appear on the very first day and the first weeks would be entirely microbial. Animals and plants would appear only in the last 4 days, with humans making their entry only in the last 30 minutes of the very last day [44]. Since bacteria were here long before us, we have been co-evolving and interacting with them from the beginning of our existence [45].

Thus, this complex symbiosis and its development are probably dependent on interactions between host–microbe genetics and the environment [46, 47]. Although the exact composition of the gut microbiota is not (yet) known, advances in metagenomic technologies have recently begun to unravel our microbial partners (the human microbiome) [48]. Ninety percent of the gut bacterias are members of two phyla (Bacteroidetes and Firmicutes), followed by Actinobacteria and Proteobacteria [46, 48, 49].

There is an increasing awareness that the microorganisms that reside within our body are part of a complex, multidirectional communication network and are in fact crucial for normal development and health. The knowledge that the bacterial content of the intestine has a significant influence on the wellbeing of the human organism was postulated even in antiquity, by the Egyptians and the Greeks. The first evidence of a treatment that aimed to change the composition of the microbiota was found in Chinese medicine during the fourth century by Ge Hong [50], famous traditional doctor who prescribed human fecal suspension for food poisoning. He called it 'yellow soup' to treat his patients with severe diarrhea. Similarly, camel stool was

also used by German soldiers to treat bacterial dysentery during World War II [51].

Later, unequivocal experimental studies consisting of comparing the physiology of germ-free (i.e. that have no microorganisms living in or on them) *versus* conventionalized animals (meaning animals that have been inoculated with the microbiota of (un)healthy animals or humans) have helped demonstrate that the gut microbiota may help to regulate host health.

Microbial dysbiosis (i.e., a disruption of the microbiota homeostasis which can include an alteration in the relative proportion of benevolent microbes to pathogenic ones) may contribute to the onset of several disorders, whereas increased microbial diversity and genetic richness are predominantly associated with a beneficial impact [52, 53]. Moreover, dysbiosis also consists of changes in the microbial metabolism, which consequently change the release of metabolites (e.g., short-chain fatty acids, bile acids, phenolic compounds) or bacterial components (e.g. lipopolysaccharides (LPS), peptidoglycans) that act on host gut key functions (gut barrier, gut endocrine function, gut immunity) [54].

Though the term 'dysbiosis' has become common in microbiome research, it remains somewhat controversial, as it implies a divergence from a healthy gut microbiota. However, the 'normal' gut microbiota cannot be easily defined as it is influenced by pleiotropic factors such as nutrition, medication, gastro-intestinal surgery, smoking, age, gender, and the area of living, plus stress, physical activity levels and other environmental factors. It is generally recognized that the intestinal microbiota of healthy adults is characterized by high subject specificity and stability over the years [55] and only undergoes temporary minor changes upon fluctuations of environmental or dietary habits. However, there are important variations in the normal intestinal microbiota from one person to another. The indigenous microbiome plays an

important role (positive or negative) in the regulation of systemic inflammation[56]. This has been particularly studied in the context of nutritional disorders, such as obesity and related metabolic disorders (i.e., including diabetes, NASH [57], cachexia, or alcohol dependence [58, 59]).

2.3 The gut microbiota and intestinal surgery

The first experimental study to demonstrate the influence of the microbiota on intestinal anastomotic healing was performed over 70 years ago, in 1955, when Cohn [60] managed to prevent anastomotic leak in a dog model by instilling daily intraluminal antibiotics through a catheter, placed just upstream of the site of a bowel anastomosis that was rendered ischaemic by ligation of the mesenteric vessels. All the dogs who didn't receive antibiotic had an anastomotic leak, the others didn't.

A decade ago, a review introduced a novel approach in which focusing on the gut microbiota could present new directions for the management of post-surgery outcomes. Indeed, the microbiota could modulate the restoration of the gastro-intestinal tract functions [61] during the postoperative period and could also impact wound healing, even at sites outside the gut.
In specific situations, such as intestinal surgery, the gut bacteria are still considered a putative source of pathobionts that can lead to further infections. Thus, mechanical bowel preparation, aiming at removing as much bacteria as possible, is still used. This procedure has an obvious, immediate impact on the composition of the gut microbiota, but it also alters the quality and the production of the protective mucus layer, which when compromised can result in eventual bacterial translocation.

Striking experimental data [62] obtained in rodents have shown that gut microbes play a crucial role in the vascularization of the intestinal mucosa

and in wound healing processes, as Cohn already demonstrated in 1955 with his experiment [60]. Moreover, gut bacteria produce numerous metabolites that contribute to the regulation of immunity and the renewal of epithelial cells and create an overall environment that limits pathogen development. Experimental and clinical trials show significant changes in the anastomotic tissue-associated microbiota as a result of changes in local environmental conditions. These changes can have either virulence potential or a beneficial effect depending on how the bacterial community composition is affected. Consequently, the benefits of bowel preparation are currently a matter of debate.

3 Physiology and the stages of anastomotic healing in relation to microbial interaction

Although clinicians still often consider it an obstacle to proper healing, the microbiota present in the wound may also have positive effects. Certain common components of the microbiota, especially butyrate-producing bacteria, assist in epithelial repair [63]. Butyrate is a key short-chain fatty acid (SCFA) considered to be a primary energy source for colonocytes and responsible for cell proliferation. This bacterial metabolite supports epithelial cell viability and barrier integrity and can downregulate proinflammatory cytokines[64]. Mucus layer-attached bacteria are usually considered more host-friendly than luminal bacteria [65, 66]. It is commonly accepted that the intestinal mucosa acts as a physical barrier, defending against bacterial invasion by forming a biofilm monolayer with intercellular contacts that prevents the translocation of pathogens (bacterial products). Other mechanisms contribute to the protection against bacterial invasion [67, 68].

These mechanisms include gut motility, which prevents prolonged contact between bacteria and potential mucosal defects; chloride secretion; cell-cell contacts; secretion of mucin by goblet cells; cytokines; and the production of antimicrobial peptides and defensins. However, after a colonic surgical intervention, several of these mechanisms are temporarily disturbed. For example, the gut motility is ineffective because of the transitory postoperative ileus that usually occurs.

Li et al. showed that the protection against mucosal infections provided by the mucus layer is complemented by the endogenous microbiota that fills the microbial niche in which enteric pathogens and opportunistic pathobionts are competing to grow and generate infections [69]. The same study further indicated that some bacterial mutants could not metabolize mucopolysaccharides and therefore act as weak colonizers compared with their parent strain. This implies that the bacterium's survival or extinction in the host may be determined by its adaptation to life in the microbial community structure in this layer. Finally, the mucus layer is very dynamic and is subject to rapid renewal. Microbes have to adapt to maintain their place in such a system, while at the same time competing with one another for resources to survive within this challenging habitat.

Different probiotics and next-generation beneficial bacteria have been associated with the improving and maintaining of a protective mucus layer [70]. Adherent *Lactobacillus spp*, are known for example, to influence the mucus barrier by increasing the expression of mucin genes, stimulating though the MUC3 expression in human intestinal epithelial cells and MUC2 production and secretion [71, 72]. Similar examples of beneficial probiotica for the mucus layer is the case of *Bifidobacterium longum*, which, when supplemented in mice after 4 weeks of feeding with a WSD restored mucus growth [73], and *Lactobacillus reuteri*, which showed a protective effect against

dextran sulfate sodium treatment in mice, increasing the mucus layer thickness [74].

Another next-generation beneficial key bacteria modifying the mucus layer is *A. muciniphila.* It was seen that during high-fat diet feeding, the mice have a thinner inner mucus layer than the control group, while in mice treated with an high-fat diet plus viable *A. muciniphila* supplementation, the opposite effect is observed (ie, a restored mucus layer thickness) [66] by inducing an increase number of goblet cells and the production of antimicrobial peptides, such as Reg3γ and LyZ1. Altogether, these findings suggest that *A. muciniphila* communicates with host cells and eventually stimulates the production of mucus [75, 76].

3.1 The process of anastomotic healing

The process of tissue repair kicks in immediately after the anastomosis (Box 1).

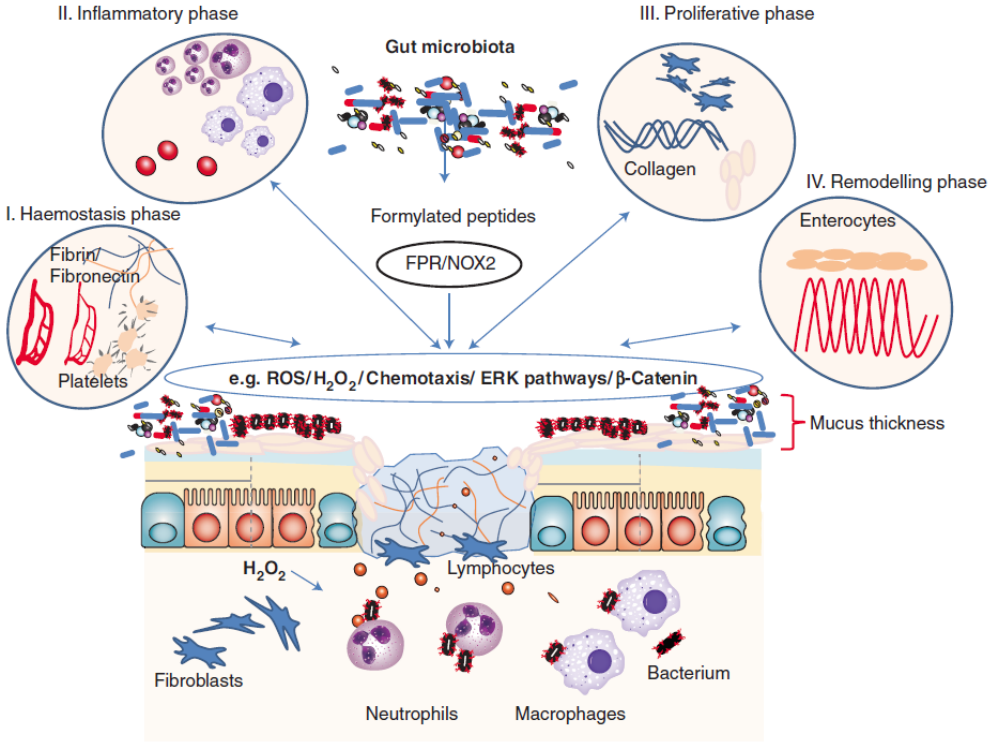
Box 1: Wound Repair / Process of healing

I. Clotting, coagulation and hemostasis
II. Inflammation:
    - Early inflammatory phase
    - Late inflammatory phase
III. Proliferation
    - Fibroblast migration
    - Collagen synthesis
    - Angiogenesis and granulation tissue formation
IV. Wound remodeling with scar tissue formation

Although it is a continuous process, it may be arbitrarily divided into four chronological phases of tissue repair (Figure 4): first comes the haemostasis

phase, during which the wound space is filled with a fibrin/fibronectin clot that temporarily seals and connects the two bowel ends. Second, the inflammatory phase, which comprises an early inflammatory phase with the activation of the complementary cascade and the initiation of molecular events that lead to the infiltration of neutrophils into the wound site, which have the main task of preventing infection. Their first critical intervention is phagocytosis to destroy and remove bacteria, foreign particles and damaged tissue. In the later stages of this phase, macrophages appear in the wound and continue the process of phagocytosis. These cells are fundamental for the late stages of the inflammatory response, acting as key regulatory cells and providing an abundant reservoir of potent tissue growth factors. The last cells to enter the wound site in the late inflammatory phase are lymphocytes. Third is the proliferative phase, which is characterized by fibroblast migration, collagen synthesis, angiogenesis and granulation tissue. Fourth is the wound remodelling phase, during which the new epithelium is developed and the final scar tissue is formed (Figure 4).

Figure 4: Microbiota influence on the different anastomotic repair phases through the stimulation of ROS [8]



Microbiota influence the stimulation of reactive oxygen species (ROS) and thereby modulate the different anastomotic repair phases. Mechanisms and factors influencing the majors repair phases such as the haemostasis phase, the inflammatory phase, the proliferative phase and the wound remodelling phase. These phases are marked by the production of a fibrin/fibronectin matrix that temporarily seals and connects the two bowel ends, the activation of the complementary cascade and the initiation of molecular events that lead to the infiltration of the wound site by neutrophils, macrophages, lymphocytes fibroblast migration, collagen synthesis, angiogenesis and granulation tissue to develop the final scar tissue. Among the molecular mechanisms, formyl peptide receptors and neutrophilic NADPH oxidase (NOX2) are activated by specific formylated peptides as well as ROS production by certain taxa of the gut microbiota. This respiratory burst is a crucial reaction occurring in the phagocytes, inducing a rapid depletion of microenvironmental oxygen, resulting in a significant proliferation of anaerobic bacteria

3.2 The links between microbes and the wound repair processes

Compelling evidence suggests that the gut microbiota contribute to epithelial repair and wound healing. For instance, seminal papers have shown that

Box 2: The microbiota role in modulating the GI restitution response

Mechanisms:

- Bacterial Competition and Cooperation
- Modulating of the GI restitution response
- Supporting epithelial cell viability
- Protection against pathogen invasion and barrier integrity
- Downregulation of proinflammatory cytokines
- Cell migration
- Epithelial cell proliferation
- Angiogenesis
- Mucus production management

axenic (germ-free) mice are characterized by a lower turnover of the intestinal epithelial cells [77]. Moreover, the healing of specific defects in the gut barrier that occurs in case of inflammatory processes or mechanical injury is impaired in germ-free animals, which have epithelial regenerative responses that are greatly reduced compared with those of conventional animals [77]. In addition, a strong correlation has been identified between different microbial communities in the wound and the ultimate repair of the wound, as will be detailed further.

The healing mechanism involves the migration of epithelial cells until the gap is closed and the barrier function is re-established. These biological processes are influenced by a variety of factors derived from the gastrointestinal microenvironment, including host epithelial and *lamina*

propria cells, the dietary and non-dietary proliferation components present in the gastrointestinal lumen, and the microbiota [61]. It has been shown that the microbiota may have an important negative or positive impact on wound healing during the inflammatory phase [56] and during cellular activation and the promotion of fibrosis [78]. In fact, both bacterial competition and the cooperation of bacterial communities can either promote or repress wound healing. For example, it has been demonstrated that *Lactobacillus reuteri* strain RC-14 can inhibit *Staphylococcus aureus* infection in a rat surgical-implant model (Box 4) [79], whereas cooperation that leads to increased virulence involves siderophore-producing bacteria, such as *P. aeruginosa*, that are able to 'share' the production of iron-scavenging siderophores with non-siderophore producers [80]. These findings shown in specific models suggest the existence of competition and/or cooperation between microbes and repair, but these remain to be confirmed in humans.

The microbiota can hinder wound healing, but it might also have a beneficial effect on it. Although different than gut wound healing, surgical skin incision and suture was studied in germ-free and conventional mice. The conventional mice showed greater initial tensile strength of the wound, including higher hydroxyproline concentrations in the surrounding tissue, compared with the germ-free mice [81].

3.3 Molecular mechanism linking microbiota signaling and wound healing

Pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), are key partners in the innate immune system. The epithelial cells of the gastro-intestinal (GI) tract express multiple TLRs that recognize bacterial PAMPs (pathogen–associated molecular patterns) [82]. Therefore, TLRs have a crucial role in regulating interactions between specific microbes and the

host response [83]. Several reports suggest that they also play a major role in intestinal epithelial homeostasis and epithelial injury. However, the microbial ligands recognized by TLRs are not unique to pathogens and are produced by both pathogenic and commensal microorganisms. Rakoff-Nahoum et al. demonstrated that commensal bacteria are recognized by TLRs under normal steady-state conditions and that acute GI epithelial damage and inflammation worsened in mice lacking TLR2, TLR4, or the myeloid differentiation primary response 88 (MyD88) [83]. Altogether, these observations suggest that the direct presence or interaction with gut microbes or specific receptors of innate immunity stimulate wound restitution and response to injury.

Epithelial restitution requires active cell migration, a process dependent on a constant turnover of focal cell-matrix adhesions. Data suggest that among the other mechanistic processes, the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) may be the key factors stimulating the beneficial effect of microbes on gut renewal and repair (Figure 4).

H_2O_2 has been shown to be one of the key ROS produced during different processes, such as wound healing, vascularization and epithelial restoration. In addition, the appropriate production of ROS helps to stimulate cell attraction, migration and adhesion and immune cell activation, all of which are involved in the regulation of wound healing (Box 4) (Figure 4) [84]. As soon as the intestinal tissue is injured, the production of ROS will confer a certain resistance to wound infection and will stimulate smooth muscle and epithelial cell proliferation to participate in wound healing and tissue regeneration (Figure 4) [85].

Although the production and impact of mitochondrial ROS has been clearly shown, the mechanisms involved in the stimulation of ROS production are less understood. It has been suggested that specific microbial-derived

molecules could be implicated. For example, formyl peptide receptors (FPRs) are activated by specific formylated peptides produced by certain taxa of the gut microbiota. These FPRs are expressed in intestinal epithelial cells, which are in direct contact with the gut luminal contents. Interestingly, specific microbial formyl peptides have been shown to stimulate FPRs and to induce ROS and the chemotaxis of immune cells (Figure 4) [86, 87].

Already a decade ago, a study demonstrated that enteric resident bacteria can potentiate epithelial restitution via the generation of ROS in epithelial cells, which in turn mediates the inactivation of focal adhesion kinase phosphatases [88]. Microbiota–host epithelial cell interactions can also modulate β-catenin signalling, a key component in regulating epithelial cell proliferation [89]. Several studies have argued that that certain common members of the bacterial microbiome (*e.g., Lactobacillus* species and butyrate-producing bacteria, which have a very potent activity) can stimulate the generation of ROS in intestinal epithelial cells through FPR-mediated signalling that modulates the extracellular signal-regulated kinase (ERK) pathway, which in turn regulates epithelial cell migration and epithelium restitution (Figure 4) [90-92].

These examples strongly illustrated the need to preserve or restore the presence of specific bacteria at the mucosal level to induce proper complex homeostatic signalling and wound-healing processes (Box 3).

Box 3: Microbiota and wound healing

- Microbiota may both hinder and have beneficial effects on wound healing.
- *Lactobacillus* spp play a major role in wound healing, stimulating pro-restitutive signaling and increasing migration and proliferation
- The administration of oral antibiotics seems to have a beneficial impact on anastomosis healing
- Reactive oxygen species and formyl peptide receptors are key molecular actors in the microbiota involved in wound healing process

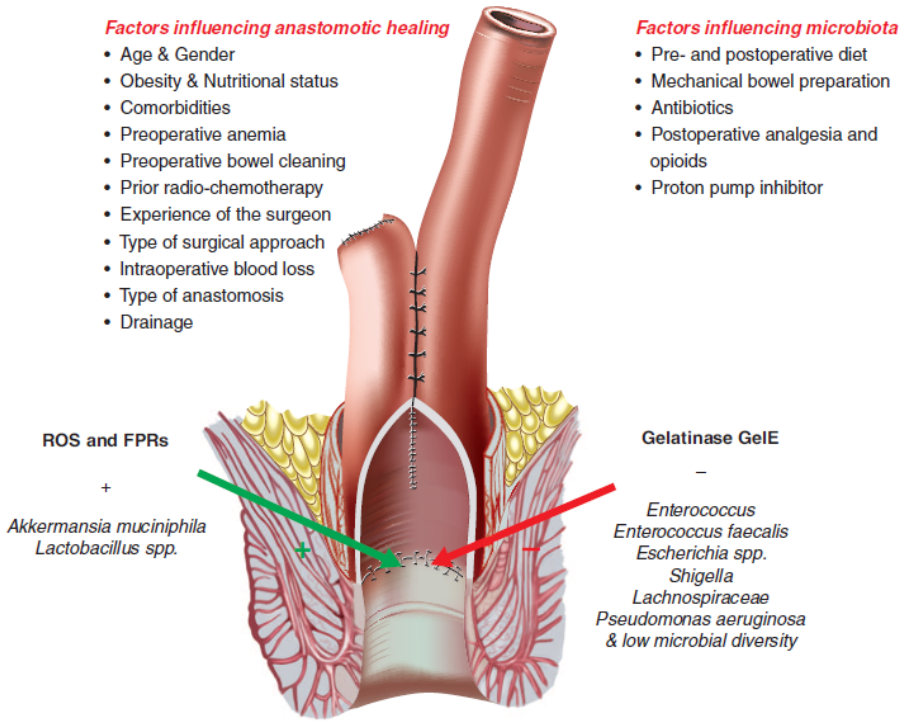


Figure 5: Risk factors for anastomotic leakage after left-sided colorectal resection.

Summary of the different factors influencing anastomotic healing and the gut microbiota composition. Mechanisms and taxa that have been associated with both positive (+, green arrow) and negative (−, red arrow) process of wound healing. Pathogenic bacteria overexploit microbiota nutrition sources and regulatory signals to promote their own growth and virulence. Bacterial proteases, that is, gelatinase (GelE), disrupt the intestinal integrity and induce increasing inflammation. FPR, formyl peptide receptor; ROS, reactive oxygen species.

4 Intestinal preparation and its impact on the microbiome

Many of the risk factors impairing the anastomotic healing have also been shown to influence the composition of the microbiota [17], while at the same time they can themselves be deeply impacted by the microbiota, suggesting the implication of the gut microbiota in intestinal wound healing[8]. Despite increasing knowledge on the matter, it is still difficult to accurately predict anastomotic leakage occurrence and, so far, there is no adequate preoperative bowel preparation that significantly helps reduce its incidence [93].

4.1 Mechanical bowel preparation—subject of debate

The use of mechanical bowel preparation (MBP) before elective colorectal procedures has been a subject of debate for more than half a century now. MBP has long been considered a surgical “dogma” and is still being used by a vast number of colorectal surgeons. In addition to the traditional effect of

the so-called “bowel cleansing”, other technical aspects argue in favour of using MBP prior to surgical intervention. These include the fact that manipulating a colon full of stool is difficult and makes the palpation of small tumours impossible. In addition, the presence of excessive stool in the recto-sigmoid prevents to carrying out a transluminal stapled anastomosis.

Since the emergence of the concept of enhanced recovery after surgery in the mid-1990s by Kehlet, the tendency to perform colonic surgery on the non-prepared colon has increased significantly without affecting the surgical outcomes. Currently, many expert centres tend to operate on unprepared colons. Still, for rectal surgery requiring a low coloanal anastomosis, which is usually protected with a temporary ileostomy, the wash-out remains the rule.

The debate was reopened following the results of three large retrospective analyses from the US published in 2015 that showed that preoperative MBP combined with oral antibiotics (OA) significantly improved the outcomes of colorectal resection[94-96]. The studies showed that this combination reduces the rate of complications after colorectal surgery by nearly half, including surgical site infection and anastomotic leak and ileus[95]. However, these findings conflict somewhat with the results of the French prospective analysis GRECCAR 3, a single-blinded, multicentre, randomized trial, completed several years ago, which analysed the postoperative results in patients undergoing sphincter-saving rectal resection for cancer without preoperative MBP[97]. For this reason, a new large international prospective multicentre randomized 4-arm trial is currently being designed and is set to begin soon. This study would randomize patients into MBP versus no MBP groups with or without preoperative OA [98]. While it is still waiting for the first results of these prospective studies which will compare all four groups separately for

the colon and the rectum surgery, two new prospective studies [99, 100], one comparing MBP+OA versus no BP and the other one comparing MBP+OA versus MBP alone suggested the usefulness of the selective decontamination of the digestive tract (SDD). These Finnish and Dutch studies were recently reviewed by Alverdy and Hyman in a paper symbolically entitled "*Bowel preparation under siege*" [101]. Although a great number of randomized trials show the clear advantages of preparation with oral antibiotics, this practice has not been adopted in routine clinical practice; the actual standard is still the use of intravenous antibiotics just before the procedure begins. The purpose of the administration of the prophylactic intravenous antibiosis is to contain the bacteria which passed the intestinal barrier in the blood to avoid the development of septicemia, whereas the administration of oral antibiotics is acting already in the intestine.

4.2 Bowel cleansing products

The efficacy of bowel-cleansing products and their safety to the patient have been studied extensively; however, the effects of lavage on the colonic microbiota have been poorly characterized to date [102].

In practice, polyethylene glycol (PEG) solutions are preferable to saline alternatives because they are easier to administer, more comfortable and better tolerated to the patient and lead to fewer complications. However, the PEG solutions for MBP result in moderate to severe loss of superficial mucus, which could also affect the composition of colonic microbiota (Box 4). These microbiota modifying properties are related to several factors. The ingested PEG electrolyte solution and the increased volume of fluids flush out the luminal bacteria, introduce oxygen into the normally anaerobic

colonic ecosystem and reduce the nutrition supply for the intestinal bacteria (Figure 5)[103]. All of these rapid changes may affect the microbial ecosystem.

4.3 Impact of bowel preparation on microbiota

To date, few studies have investigated and described the impact that bowel preparation with or without oral antibiotics conducted prior to colorectal surgery may have on the mucosa-associated and luminal colonic microbiota (Table 1). Even fewer studies have reviewed the direct impact of the gut microbiota on the healing of a colorectal anastomosis (summarized in Table 1).

Box 4: Microbiota and bowel preparation

- Single-dose MBP (2 L) has a more severe effect on the microbiota than a double split dose (two separate 1L dosages) does.
- PEG solutions for MBP result in an important loss of superficial mucosal mucus, which affects the microbial ecosystem.
- Almost *restitutio ad integrum* of the microbiota occurs a few weeks after MBP in healthy patients.
- A 24-hour clear liquid diet does not change the microbiota in the short term.

Table I: Studies conducted to verify the effect of bowel preparation on the microbiota composition

Type=Type of bowel preparation: polyethylene glycol solutions (PEG) or saline solutions (NaCl)

M vs. L: Mucus associated bacteria versus luminal content bacteria
Met = Metabolomic Analysis

	Year	N	Type	M vs L	Design of the study	Results
Mai. V et al.[104]	2006	5	-	L	Comparing intraluminal microbiota precolonoscopy, shortly after colonoscopy and 6-8 weeks after colonoscopy using PCR-DGGE (denaturing gradient gel electrophoresis)	MBP has a significant effect on the luminal gut bacteria, with NO clear trends detected in pre- and postcolonoscopy samples

Bucher P. at al [105]	2006	50	PEG	M	Prospective randomized study of 50 patients who did or did not receive MBP before left-sided colorectal surgery for benign or malign disease with the purpose to determine whether mechanical bowel preparation is associated with histologic alterations in the colon All patients received iv antibiotics prophylaxis	The most striking alterations associated with MBP were the loss of superficial mucus and epithelial cells. In addition, inflammatory changes, *i.e.*, lymphocytes and polymorphonuclear cell infiltration were more prevalent after MBP
Harrel L. et al.[106]	2011	12	PEG	M	Mucosa-associated microbiota analysis via biopsies carried out during sigmoidoscopy. The subjects were split into 3 groups to compare the impact of MBP or liquid diet for 24 hours with no preparation	Changes in the microbiota composition after MBP and no changes in both control groups were observed Reduction in overall mucus-associated microbiota diversity after MBP Changes in the microbiota composition at the genus level but not at phylum level were found

O' Brien C. at al [107]	2013	15 +5	PEG	L	Analysis of microbiota in stool samples collected 1 month and 1 week before and 1 week, 1 month and 3 months after by PCR-DGGE and HTS (high throughput sequencing)	Short-term nonspecific changes in the microbiota with no lasting effect on the composition of the intestinal microbiota
Jalanka J. et al. [108]	2015	23	PEG	L	Randomized study analysing the microbiota in 2 groups that consumed a bowel preparation treatment (Moviprep) either in two separate doses of 1 L or as a single 2 L dose	Immediately after the lavage, the amount and composition of the microbiota altered drastically Restoration of the bacteria levels and community composition within 14 days Consumption of the purgative in a single dose had a more severe effect on the microbiota composition than that of a double dose and notably increased the levels of *Proteobacteria*, *Fusobacteria* and bacteria related to *Dorea formicigenerans*

Drago L. et al. [109]	2016	10	PEG	L	Study of the microbiota composition immediately after MBP and 1 month thereafter	MBP has a long-lasting effect on the gut microbiota composition and homeostasis, with a particular decrease in *Lactobacillaceae*, a population of protective bacteria
Shobar et al. [110]	2016	18	½ PEG ½ NaCl	L+ M	The effect of bowel preparation on the mucosa-associated and luminal colonic microbiota in healthy subjects and inflammatory bowel disease patients by harvesting the sampling during an unprepped sigmoidoscopy and 1 week later during a prepped colonoscopy using HTS	MBP massively affected the composition and diversity of the faecal and luminal microbiota in the short term Luminal samples were similar to mucosal samples immediately post MBP Both the luminal and mucosal compartments of the gut microbiota are affected

Nagata N. et al. [111]	2019	31	½ PEG ½ NaCl	L+ Met	Comparing a bowel prep group (n=8) with a control group (n=23). Analysis of luminal colonic microbiota collected one day prior BP as well as 1- and 14 days after. Additionally, metabolomic analysis on the samples collected from the BP-Group	Microbiota composition was significantly reduced immediately after the prep but not at 14 days after it. 32 metabolites were significantly changed with the prep, but these metabolites recovered within 14 days Overall composition recovers to baseline within 14 days

Mai et al. were the first to examine the effect of bowel preparation and colonoscopy on the intestinal microbiota composition [104]. Bucher et al. analysed the differences in the stool microbiota in patients who did or did not undergo preoperative MBP after left-sided colorectal surgery for benign or malignant disease [105]. Harrel et al. split the subjects into three groups to compare the impact on the microbiota of MBP or a liquid diet for 24 hours with no bowel preparation (Box 4) [106]. O'Brien et al. analysed the microbiota in stool samples collected one month and one week before and one week, one month and three months after MBP using denaturing gradient gel electrophoresis (DGGE) and high-throughput sequencing (HTS) methods [107]. Jalanka et al. were the first to examine the effect of two cleansing regimens on faecal microbiota composition [108]. This was also the first study to analyse the different effects associated with two different methods for administering the PEG solution (Moviprep): as separate 1-litre doses or as a

single 2-litre dose (Box 4). Drago et al. compared the microbiota composition immediately and one month after MBP; contrary to most other studies, they found that MBP has long-lasting effects on the gut microbiota composition and homeostasis, particularly in terms of a decrease in the abundance of *Lactobacillaceae*, a population of protective bacteria (Figure 6) [109]. Shobar et al. conducted one of the most thorough studies, which simultaneously analysed the composition of the microbiota at the levels of the mucosal and luminal compartments, differentiating between healthy subjects and patients with inflammatory bowel disease (IBD) and using a high resolution technique [110]. Nagata et. al were the first to study, beside the changing of the intraluminal gut microbiota post MBP at 1 and 14 days by 16S rRNA sequencing, also the metabolome compositions by using capillary electrophoresis time-of-flight mass spectrometry [111]. They showed that at the genus and species level, compared with controls, microbiota composition was significantly reduced immediately after the prep but not at 14 days after it. In addition, for the gut metabolome profiles, correlation coefficients between before and immediately after the prep were significantly lower than those between before and 14 days after prep and were not significantly different compared with those for between-subject differences. Thirty-two metabolites were significantly changed before and immediately after the prep, but these metabolites recovered within 14 days.

As summarized in Table 1, all of the studies clearly showed a massive impact on the diversity of the gut microbiota composition in the colonoscopy samples taken after MBP. Moreover, MBP does not decontaminate the GI tract as the number of bacteria remains almost the same as when MBP is not performed. Finally, the most deleterious effect of MBP is that it disrupts the microbiota enough to change its composition, decreasing the diversity and allowing opportunistic pathogens to thrive [112]. In contrast, in healthy

subjects, an almost complete restitution of the microbiota composition was noted just a few weeks after MBP (Box 4).

All studies describing the modification of the gut microbiota immediately after the MBP showed important short-term distortions in the composition and an important reduction in diversity without being able to determine one unique general pattern. Most often, they indicated an increased level of Proteobacteria and Bacteroidetes, a significant reduction in *Lactobacillaceae*, an increase in *Enterobacteriaceae* abundance at the family level and a drastic change in the ratio of Gram-positive to Gram-negative species. Similar changes were observed when studying the microbiota in diarrhoea diseases in general (Figure 6) [113].

Purging also leads to the introduction of oxygen into the normally anaerobic colonic ecosystem, resulting in an increased amount of Proteobacteria. In addition, the observed increase in pH might reflect the loss of specific bacterial metabolites, such as SCFA, thereby allowing Proteobacteria to flourish [108].

4.4 Impact of antibiotics use on intestinal anastomotic healing

The discovery and introduction of antibiotics was a huge step forward in colorectal surgery. The first experimental study to demonstrate the influence of the microbiota on intestinal anastomotic healing was performed over 70 years ago, when Cohn et al. managed to prevent anastomotic leak in a dog model by instilling intraluminal antibiotics (i.e., achromycin) at the site of a bowel anastomosis that was rendered ischemic by ligation of the mesenteric vessels [60]. Interesting findings have also been made with regards to the

impact that antibiotics with MBP may have on the microbiota. Not all commonly used oral antibiotics appear to have positive effects [114]. Antibiotics that induce bacterial translocation have been found to result in increased inflammation in response to coincident epithelial injury [115]. It is well established that excessive uncontrolled inflammation results in a variety of pathological conditions, including, at the extreme, septic shock.

Conversely to MBP, the administration of antibiotics with broader coverage than necessary provokes an immediate and long-lasting effect on microbial diversity (Figure 6), often leading to incomplete recovery and increased antibiotic resistance [116]. A significant reduction in Bacteroidetes and a concurrent increase in Firmicutes was found after the administration of broad-spectrum antibiotics [117]. Products containing probiotics (beneficial microorganisms), prebiotics (substrates that enrich beneficial bacterial populations) or synbiotics (combinations of both), aiming at increasing the number and activities of beneficial gut microbes, will most likely comprise an alternative or complementary approach to non-selective antibiotic therapy in the near future.

The same considerations must also be applied with regards to colon cleansing procedures performed independently from a medical intervention. Claims of marked health benefits from “detoxification” have stimulated public interest in colonic cleansing and a whole industry has developed on this premise. However, very limited scientific research is available regarding the impact of these “colon detox” procedures designed to empty the bowel. It is very likely that they lead to imbalances in the microbiota with adverse health effects.

At present, very little is known about how to re-establish the microbiota after sudden iatrogenic modification. The question of whether the humans possess a distinct source or niches for re-establishing normal microbiota is still to be answered. The appendix has been suggested to represent a ‘safe

house' for gut microbes [118]. Gut-associated lymphatic tissue is present in the appendix, suggesting that is has important immune functions for regulating microbiota composition. In contrast, inflammation in the appendix has been associated with local invasion by *Fusobacterium nucleatum* [119]. However, it is difficult to conceive that all patients without an appendix will develop serious problems with recovery after a bowel preparation. It is more likely that the real drivers of partial recovery of the initial ecosystem are the restoring forces within a dynamic system rather than passivity.

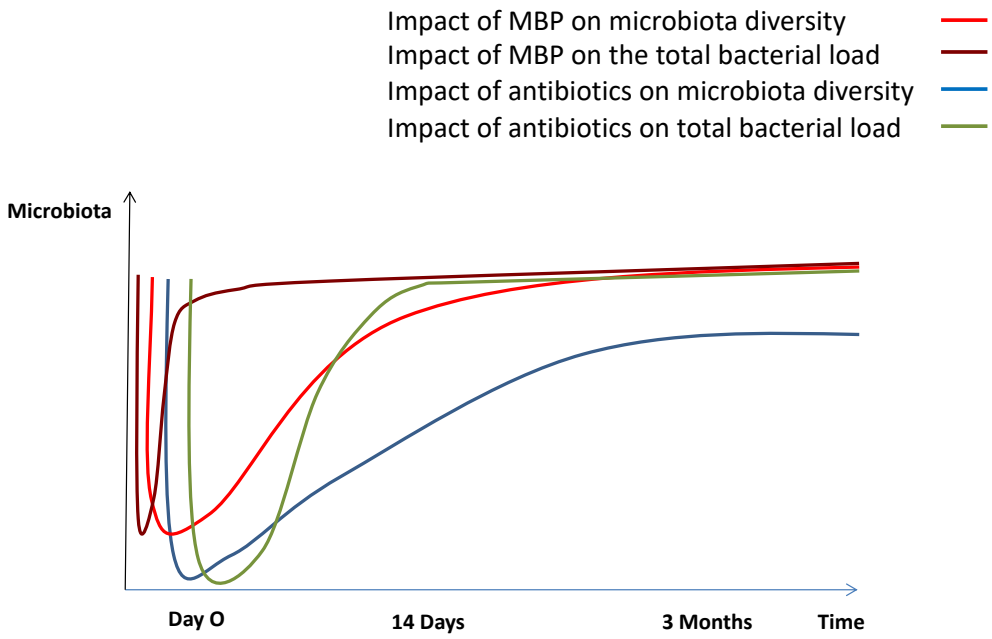


Figure 6: The Impact of MBP and Antibiotics on Microbiota Diversity and Total Bacterial Load

The curves which are based on the very few, quite heterogenic studies converge towards a significant decrease of the total bacterial load as well as of their diversity with a fast recovery of the absolute bacteria number and a regeneration of the diversity almost ad integrum within two weeks after the MBP. Antibiotic preparation

leads to an almost complete drop of the total bacterial load with a similar fast recovery of the total number of bacteria but with a very slow, incomplete regeneration of the diversity, even months after and most probably with permanent consequences [116]

In summary, to this date and in the light of all the evidence available so far, there is no clear answer to the question whether interventional gut preparation should be performed prior to surgery or not.

Recent research shows that the gut microbiota acts as an ecosystem with a very important role in the well-being of the entire organism. It has also been shown that the microbiota has a great influence on the healing process of an intestinal anastomosis.

Compelling research proves that standard bowel preparation with or without the administration of antibiotics disturbs the diversity and the fragile balance of the normal and putatively healthy microbiota. This disturbance is associated with an immediate impact on the gut barrier function and changes in intestinal innate immunity. The imbalances caused by the preparation methods may have an impact on the healing process of the anastomosis, thereby calling their supposed benefit into question. At the same time, there are no proven alternatives to the use of antibiotics, even though its well-proven positive effects on healing go hand-in-hand with the disturbance of the microbiota.

In addition, as Alverdy [101] recently commented, the most frequently used 'intravenous' prophylactic antibiotics for colonic surgery are cefazolin (first-generation cephalosporin) or cefuroxime (second-generation cephalosporin) associated with metronidazole. Today, the most common 'oral' antibiotic regimen used is neomycin and metronidazole, the microbiological basis of which remains unverified. It is unclear why both oral and intravenous

metronidazole are administered simultaneously given that oral metronidazole is nearly completely absorbed systemically in the proximal small bowel[120]. Deierhoi[121] showed that the choice of antibiotics in colonic surgery matters greatly in terms of preventing surgical site infections. Despite these findings, a one-size-fits-all antibiotic regimen in colonic surgery appears to have become a standard procedure, with cefazolin/ cefuroxime and metronidazole being used worldwide prophylactically as the most common intravenous antibiotic regimen at the induction in colorectal surgery.

5 Novel insights into the role of specific microbiota and techniques in colorectal surgery

Novel techniques to improve some of the factors that might influence the wound healing in a reasonably short period have been tested in rodent studies and ongoing clinical studies but outcomes have been either inconclusive or disappointing but the successes are relatively limited. The incidence of anastomotic leak after colorectal resection varies from 1% to 19%, depending mostly on the anastomosis localization; the highest leakage rate of up to 24% [122] was observed in low colorectal or coloanal anastomosis (Figure 2). Anastomotic leak is associated with an increased risk of morbidity, short-term mortality and permanent ostomy [123]. Furthermore, in cancer patients, anastomotic leak has been associated with increased local recurrence and poor survival after colorectal cancer surgery [124]. In recent decades, the rate of incidence of colorectal anastomotic leaks has remained constant, despite significant technical surgical advances, like the use of novel surgical devices, improved staplers, laparoscopic- or robotics-guided

procedures or even perioperative arterial vascular control by indocyanine green-enhanced fluorescence-guided colorectal resection).

The benefits of using fluorescence angiography to control vascularisation of the anastomosis after intestinal resection were clearly demonstrated in the PILLAR II study [125]. By using the indocyanine green-enhanced (ICG) fluorescence the frequency of anastomotic leakage dropped to less than 2%. More recently published data confirmed this finding. According to the work of Cohn et al. [60], Reinhardt et al. and others [62] the microbiota induced vascular remodelling is of huge importance for the adequate healing of an anastomosis.

A potential way to reduce the impact of perioperative microbiota disturbance would be the implementation of the enhanced recovery protocol (ERP) which was developed to reduce physiological stress and postoperative organ dysfunction after surgery. This protocol, also known as “Fast-Track”, gained significant adherence of many surgical teams in the last 20 years. It is based on pre-, intra- and postoperative measures, e.g. avoidance of MBP, use of minimal invasive surgery, avoidance of perioperative fasting, standardized anesthesic and analgesic regimens and early mobilisation.

The ERP has been shown to significantly improve the morbidity and mortality. Although so far data are lacking, it can be assumed that this protocol might also reduce negative influence on the loss of microbiota diversity and specificity by promoting a safe postoperative recovery with early return of bowel function.

At present, only a few studies have examined the impact of an intestinal anastomotic injury on the microbiota composition. More scientific efforts should be made to determine whether a specific dysbiosis resulting from surgery trauma or an eventual bowel preparation might favour the development of anastomotic leakage.

In clinical routine, the effect of an intestinal anastomotic injury on the microbiota is likely not caused by surgical stress alone but is probably the result of the perioperative management as a whole. In consequence, there are many potential factors that may influence the microbiota to take into account. We may cite, for instance, MBP, the use of antibiotics, modulation (or lack thereof) of the pre- and postoperative diet, the use of postoperative analgesia and opioids, and the administration of proton pump inhibitors or any gastric acid reducers (Figure 5). Additionally, transanal drainage tube placement or the use of vasoactive medicines are also specific confounding factors (Figure 5). Of course, the initial composition of the gut microbiota might already be dysbiotic and therefore not "normal" because of the specific disease to be treated. For example, there is little doubt that the microbiota of IBD patients are very distinct from those of healthy subjects [110]. Even though data are scarce for the moment, it is reasonable to assume that other diseases for which a colorectal surgery will ultimately be required, such as repeated diverticulitis or colorectal cancer, are also characterized by changes in the microbial diversity and also prone to induce a chronic inflammatory status. As recently shown by Barbara et al. [126] these changes threaten the microbiota diversity and allow pathogenic bacteria that contribute to postsurgical complications to thrive. Hence, priority should be given to stimulating further research to identify clinical viable solutions to ameliorate the preoperative microbiota.

A number of studies have described bacterial wound communities, especially in chronic open wounds, and they reported the following observations [127-131]

- The number and proportion of bacterial species can range greatly among individual wounds.
- Bacterial diversity determined with culture-based methods is

significantly lower than that determined with new culture-independent methods, such as PCR amplification of the 16S rRNA gene.

- The microbiota can differ among different wounds, while the bacterial communities at different sites within an individual wound are significantly more similar to one another than to those of different wounds [127].
- The reliability of both culture- and non-culture-based analysis depends heavily on the sampling method used. For example, certain sampling techniques will not detect anaerobic bacteria, which are common in chronic wounds [132]. Therefore, when studying the human microbiota, important controls need to be assured to guarantee that the chosen sampling techniques are as unbiased and comprehensive as possible.

Shogan et al. analysed the changes in luminal versus tissue-associated microbiota at anastomotic sites created in the colons of a rodent model using 16S rRNA gene amplicon sequencing of samples collected on the day of surgery and the 6th day following surgery [133]. They did not use antibiotics either pre- or postoperatively, and they did not perform bowel preparation. The results indicated that anastomotic injury induced significant changes in the anastomotic tissue-associated microbiota with minimal differences in the luminal microbiota (stool).

A 500-fold and 200-fold increase in the relative abundance of *Enterococcus* and *Escherichia/Shigella*, respectively, were identified as the most significant differences. Functional profiling predicted the prevalence of bacterial virulence-associated pathways in postanastomotic tissues, including the production of hemolysin [134, 135], cytolethal toxins [136], cytotoxic necrotizing factor [137], bacterial attachment to mammalian cells via AIDA-I

adhesin-like protein, cytotoxicity to eukaryotic cells as a result of cytotoxic necrotizing factor [137], increased motility (outer membrane usher protein, fimbrial-like protein, type 1 fimbrial protein, fimbrial chaperone protein) [138], and invasion of epithelial cells that can be attributed to *Enterobacteriaceae*, particularly the *Escherichia* genera [139]. Proteolytic cleavage by coccolysin, also known as gelatinase, can be attributed to *Enterococcus* [134, 140]. Importantly, the gelatinase GelE of *Enterococcus faecalis* has been shown to degrade collagen, a critical component of anastomotic tissue healing (Figure 5) [141]. Although little is known about *Enterococcus faecalis* and its possible influence on leakage, this bacterium is highly prevalent in leaking anastomotic tissues [142, 143]. *Enterococcus* has a high adherence affinity to extracellular cellular matrix proteins such as fibronectin, laminin, and various different types of collagens [144-146], including collagen IV [147].

In a clinical study, Komen et al. proposed a practical fast-screening tool for the early detection of colorectal anastomotic leakage based on the presence of *Escherichia coli* and *E. faecalis* in drained fluid using RT-PCR [143]. They showed that the increased presence of *E. faecalis* in drained fluid may be a sensitive screening tool for the early detection of colorectal anastomotic leakage, while negative test results virtually rule out the presence of an anastomotic leak.

As previously mentioned, age is an important risk factor for colorectal surgery. Interestingly, it has been shown that the microbiota composition at the extremes of life, in infants and the elderly, is different from that of adults [148]. The core microbiota of elderly subjects is distinct from that previously established for younger adults, with a greater proportion of *Bacteroides* spp. and distinct abundance patterns of *Clostridium* groups [149], which may contribute to the known increase in the anastomotic leakage risk among the elderly.

A critical loss of cytoprotective microbiota in anastomotic tissues may further facilitate colonization and invasion by disease-associated microbiota, again with the potential to complicate healing (Figure 5). Levels of the commonly cytoprotective bacteria belonging to the family of *Lactobacillaceae* have been also shown to be reduced in anastomotic tissues [150-152]. The lack of such taxa may play a role in the development of leaks when levels are critically lowered and additional physiologic stress occurs. As described earlier, these taxa contribute to wound healing and repair via mechanisms involving ROS production and specific formylated peptides acting on FPRs (Figure 4) [153,154]. Nevertheless, in the study by Okada et al. , conventional rats had a significantly higher anastomotic bursting pressure in the ileum compared with both rats whose colons had been mono-contaminated with *Lactobacillus acidophilus* and with germ-free rats, suggesting the importance of a whole bacterial community and the interactions within the community [152].

In a 2002 rodent study, Stappenbek et al. demonstrated the key role of the microbiota in (re)constructing the mesenchymal microvascular network through the intermediary of Paneth cells, a key cellular component of the innate immune system. They showed that *Bacteroides thetaiotaomicron* stimulates angiogenesis by doubling the capillary network in the small intestine via Paneth cells [155].

In 1997, Schardey et al. suggested that *Pseudomonas aeruginosa* might play a causative role in anastomotic leak [156] and performed the first randomized, prospective, double-blind and placebo-controlled trial with antibiotics, confirming that microbes played a role in human anastomotic leak [157]. In this trial, patients received either placebo or decontamination with oral antibiotics just before and one week after the surgical intervention. All of the patients received perioperative intravenous antibiotic prophylaxis. The authors concluded that this decontamination during anastomotic wound healing is

safe and effective for preventing esophago-jejunal anastomotic leakage after total gastrectomy.

In another study, a novel model of anastomotic leakage was developed in which rats that had been preirradiated (as is usually necessary in rectal surgery) underwent distal colon resection and then were intestinally inoculated with *P. aeruginosa*, a common colonizer of the radiated intestine [158]. A significantly higher incidence of anastomotic leakage was found when the rats were colonized by *P. aeruginosa* compared with radiation alone. Moreover, the study showed that the inoculated strain mutated in cases of anastomotic leakage, showing altered pyocyanin production and enhanced collagenase activity, high swarming motility, and destructive activity toward cultured intestinal epithelial cells (i.e., apoptosis, barrier function, cytolysis). In contrast with the radiated rats, the non-radiated rats maintained a normal microbiota and an intact mucus layer, which may shield against the virulent effects of the mutated phenotype. This work demonstrates that the in vivo transformation of microbial pathogens into a tissue-destroying phenotype may have important implications in the pathogenesis of anastomotic leak.

Finally, the use of the novel virulence-suppressing compound PEG/Pi *(*high-molecular-weight polyethylene glycol*)* prevented *P. aeruginosa* from transforming to the tissue-destructive phenotype and prevented anastomotic leak in rats [159]. Wu et al. demonstrated that PEG/Pi has the potential to function as a surrogate mucin within the intestinal tract of a stressed host by inhibiting key interactive events between colonizing microbes and their epithelial cell targets [159].

Van Praagh et al. conducted a unique clinical study that investigated the microbiota composition in the area of anastomotic fistulas. The study was conducted with patients included in the C-seal trial. The C-seal is a

biodegradable sheath that protects the stapled colorectal anastomosis from leakage [160]. The researchers compared the mucosa-associated microbiota collected at the anastomosis level during the surgical intervention from 8 patients who developed anastomotic leakage requiring re-intervention with that of matched individuals from the same trial who had a favourable evolution [161]. They found a correlation between the bacterial family *Lachnospiraceae*, low microbial diversity and anastomotic leakage, possibly in association with the BMI. The relative abundance of the *Lachnospiraceae* family (phylum Firmicutes) may be explained by the higher abundance of mucin-degrading Ruminococci (phylum Firmicutes) within that family in anastomotic leakage cases, which is also observed in IBD (Figure 5).

The studies discussed above suggest that commensal microbes are influenced by intestinal resection and anastomosis, along with other aspects of perioperative management. This results in the bloom of specific species that are normally present in the normal commensal microbiota (i.e., *Enterococcus* and *Pseudomonas*) but that display a more aggressive, tissue-destroying phenotype. This could contribute to the pathogenesis of anastomotic breakdown and clinical leakage [162].

6 *Akkermansia muciniphila:* Origin, mechanism of action

Akkermansia muciniphila (Figure 7) is one of the most abundant species in the human intestinal microbiota (0.5–5% of the total bacteria). It has been isolated and characterized as a mucin-utilizing anaerobe in 2004 by Prof. Willem de Vos [163, 164] and Dr Muriel Derrien during her Ph.D. research at

Wageningen University. Its discovery was initiated by the notion that the human body produces its own microbial substrates or “prebiotics”, namely mucus, an abundant glycoprotein that is specifically produced and degraded in the colon [165].

Figure 7: The “Identity Card” of *Akkermansia muciniphila*

Akkermansia is a gram-negative bacterium that has been isolated by Pr. Willem de Vos and Muriel Derrien in 2004. Its importance for colon surgery derives from the fact that it lives in close vicinity with the colonic wall and, more specific, at the level of the mucus. In other words, this bacterium is also considered as a mucus specialist because it ‘feeds’ on the mucus and uses the degraded mucin for energy, being associated with a thicker mucus layer; it sends signals to host epithelial cells to produce more mucin and thus is restoring and improving the gut barrier function and reduces inflammation. It is present in relatively high abundance, between 0,5-5 % of the entire microbiota community in healthy subjects. Since its discovery, evidence is accumulating suggesting a beneficial role for A. muciniphila [45 ,166, 167].

6.1 Isolation of *A. muciniphila* and the abundance fluctuations in adults

Akkermansia muciniphila was isolated using a strategy specific for the isolation of dominant bacteria: dilution to extinction (i.e. isolation based on serial dilutions). This led to the isolation from a healthy adult of a new species from a novel genus within the *Verrucomicrobia* that was termed *Akkermansia muciniphila* and was represented by the type strain Muc^T [168].

A. muciniphila was found to be an exclusive mucin-degrading specialist, present in the human intestine since early life in all tested subjects[169]. Recent comparative growth analysis showed *A. muciniphila* to be the most active isolated utilizing mucin *in vitro* [170]. *A. muciniphila* Muc^T was also found to be capable of utilizing glycans human milk oligosaccharides (HMOs) [164, 171, 172] probably explaining its presence in early life. Only a few other substrates were found to stimulate its growth as pure culture, including metformin [173], betain [174] and tryptophan [175].

The rapid characterization of *A. muciniphila* has been facilitated by its relatively limited diversity and only one other species isolated from reticulated python, *A. glycaniphila,* has been described with slightly different metabolic properties [176].

Since *A. muciniphila* is virtually the only representative of the *Verrucomicrobia* in the human gut, many 16S rRNA gene sequence surveys that reported this phylum, often represented the species *A. muciniphila* [177]. A recent study analyzing over 2000 *Akkermansia* metagenomes confirmed this and showed *A. muciniphila* to be, by far, the dominant species with highly similar (over 98 % identity) 16S rRNA sequences.

Research showed that the genus *Akkermansia* appears in the first year of life and gradually increases in abundance until adulthood [164]. It has been showed that different factors influence the relative colonic abundance of *A.*

muciniphila. Amongst these, transit time [178-180], exercise [181], and intake of polyphenols-rich foods [182] have been consistently identified as influencing factors in independent studies. In addition, several reports show that caloric restriction or starvation increases the abundance of *A. muciniphila* in the gut [183-185]. Metformin appeared to be an important driver as this anti-diabetic drug can stimulate the growth of *A. muciniphila* and hence has been identified as a major confounder in studies addressing type 2 diabetes [173].

6.2 Benefits of *Akkermansia* for health

Different pathological conditions, such as metabolic disorders including obesity, type-2 diabetes (T2D), non-alcoholic liver diseases (NAFLD) and cardiovascular diseases (CVD) have been associated with a reduction in the abundance of *Akkermansia* spp. in rodent and human studies [66, 186-190]. On the other hand, different studies showed a positive correlation between the abondance of *A. muciniphila* and the treatment of different pathological situations. For example, it was found that the abundance of *A. muciniphila* in overweight and obese subjects was positively associated with healthier metabolic status as well as with better clinical outcomes after caloric restriction [191]. Furthermore, several studies show that restoring the levels of *A. muciniphila* reduces IBD, alcoholic liver disease, colitis-associated tumorigenesis, and even progeria [75, 192-197].

Further studies investigated the benefits of *A. muciniphila* abondance in cancer treatment. For example, patients with Non-Small-Cell Lung Carcinoma and Renal Cell Carcinoma that responded well to anti-PD1 antibodies (immunotherapy) showed an enrichment of *A. muciniphila* [198], with a proven amelioration of the anti-tumor effect of the immunotherapy [199].

Contrary, the prevalence of *A. muciniphila* was the lowest (34%) in subjects with rapid progression of the disease and those that later deceased.

To date, associations between the abundance of *A. muciniphila* and neurodegenerative disorders have been identified for dozens of diseases and syndromes but experimental causal relationships were found only in a few cases [200].

6.3 Mechanisms of action of *Akkermansia*

Since A. muciniphila Muc^T is known as a propionate producer in the presence of vitamin B12 [201], a possible explanation of how *A. muciniphila* is contributing to the homeostasis and the human health, could be linked to the generic effects of short-chain fatty-acids (SCFAs) such as propionate. Whereas the contribution of *A. muciniphila* to overall propionate in the gut may be relatively low versus other dominant propionate producers such as *Bacteroidetes*, notably under fasting conditions *Akkermansia* seems to be upregulated and could make a significant contribution to the production of this SCFA [202]. However, this theory regarding the mechanism of action of *Akkermansia* in relation to the homeostasis, was revised by the discovery that *A. muciniphila* maintains its beneficial properties after pasteurization [203].

Preclinical [66] and clinical [204] trials showed that the daily oral supplementation with live *A. muciniphila* has a significantly favorable outcome on the onset of obesity, diabetes and gut barrier dysfunction in mice.

In our lab, we studied further the possible mechanisms of action and knowing that the metabolic endotoxemia or bacterial translocation [205, 206] can be caused by an increased plasma LPS level, our team observed that live *A. muciniphila* prevented the development of metabolic endotoxemia, an effect associated with the restoration of a normal mucus layer thickness [66]. This

research also showed that administration of live *A. muciniphila* restored the endogenous production of antimicrobial peptides and increased the endogenous production of specific bioactive lipids that belong to the endocannabinoid family and are known to have anti-inflammatory activities and regulating the endogenous production of gut peptides involved in glucose regulation and gut barrier, respectively, glucagon-like peptide-1 and 2 (GLP-1 and GLP-2) [207]. Numerous other studies confirmed these results and also showed ameliorations in other specific disorders such as atherosclerosis, hepatic inflammation and hypercholesterolemia [75, 203, 208, 209].

In 2013, a study [66] showed that the beneficial effects of *A. muciniphila* disappeared when the bacterium was destroyed by using autoclaving, a heat treatment that destroys all the constituents of bacteria and spores. Consequently, knowing that *A. muciniphila* is a Gram-negative bacterium and hence no spore-former, our lab investigated, what would be the effects of the pasteurization, which is a milder heat inactivation method compared to autoclaving. Therefore, the lab tested the impact of administrating pasteurized *A. muciniphila* (30 min at 70°C) cells on diet-induced metabolic disorders in mice. Surprisingly, in 2017, our laboratory showed that, through the pasteurization, the beneficial effects of *Akkermansia* were maintained, and they were even exacerbated. It further discovered that the mice treated with pasteurized *Akkermansia* and receiving high fat diet, experienced significantly lower body weight gain and fat mass and that these effects were independent of the food intake, and that was, of course, without changing the propionate production. The pasteurized *A. muciniphila* increased the loss of energy in the faeces of the treated mice, indicating a decrease in energy absorption that could contribute to explaining the lower weight gain. Pasteurized *A. muciniphila* also strongly improved glucose tolerance, hepatic insulin sensitivity, and completely blocked the diet-induced metabolic

endotoxemia. Although the exact mechanisms of action are not fully elucidated, it was found that the process of pasteurization, even if it "kills" the live bacteria, increases the presence of certain proteins like Amuc_1100 which is known as one of the proteins that *A. muciniphila* express on its outer membrane [210]. Amuc_1100, is involved in the formation of pili by *A. muciniphila* [203]. For the moment, it is not known if Amuc_1100 might explain all the beneficial effects of *A. muciniphila* but its production levels per cell are sufficiently high to explain the effects in preclinical models [44] (Figure 8).

Moreover, Amuc_1100 was found to activate toll-like receptor-2 (TLR-2). The TLRs are a family of receptors comprising 10 members in human (13 in mice). Each of the TLRs mediates responses to distinct microbial components derived from pathogens. Two typical examples are TLR2, which senses bacterial lipoproteins [211] and TLR4, which recognises bacterial LPS [212]. Together the TLRs cover a wide range of both external stimuli (PAMPs) [213] and internal signals derived from tissue damage (damage-associated molecular patterns (DAMPs) [214].The administration of Amuc_1100 replicates most of the beneficial effects of *A. muciniphila* Muc^T cells, including those in specific disease models of intestinal inflammation and colonic cancer [197, 203, 215].This suggests that even after pasteurization, *A. muciniphila* Muc^T can still improve host health, eventually arguing against the need for potential secreted metabolites to observe beneficial effects of the bacterium.

Other proteins produced by the live form *A. muciniphila* Muc^T with interesting effects have been recently discovered. One example is the recently identified protein named P9 (encoded by the earlier identified Amuc_1831 gene [216]), that was responsible for the stimulation of GLP-1 *in vitro* [217]. Mice treated with an oral administration of P9 for 8 weeks at very high doses were found to have a reduction of the body weight gain, fat mass gain and increases in

the intestinal expression of proglucagon (i.e., the precursor of GLP-1). This protein is acting through ICAM-2 (Figure 8). Strikingly, it was not possible to link these effects with higher circulating levels of GLP-1. However, it was found that the pharmacological usage of P9 activates ICAM-2 and these effects were linked with possible GLP-1 secretion since anti-ICAM-2 antibodies partially abolished the effects of P9 on the GLP-1 secretion in vitro. Given that *A. muciniphila* Muc^T increases the expression of IL-6 in the ileum of the mice, besides confirming that IL-6, is a proinflammatory cytokine, this cytokine also dose-dependently increases the secretion of GLP-1 in vitro. Therefore, the authors hypothesized that *A. muciniphila* Muc^T could also act through an IL-6/GLP-1-signaling. To further support their findings, they have used IL-6 knockout mice and found that these mice failed to respond to P9 induced GLP-1 secretion whereas also blocking the GLP-1 receptor abolished the P9 effects on thermogenesis.

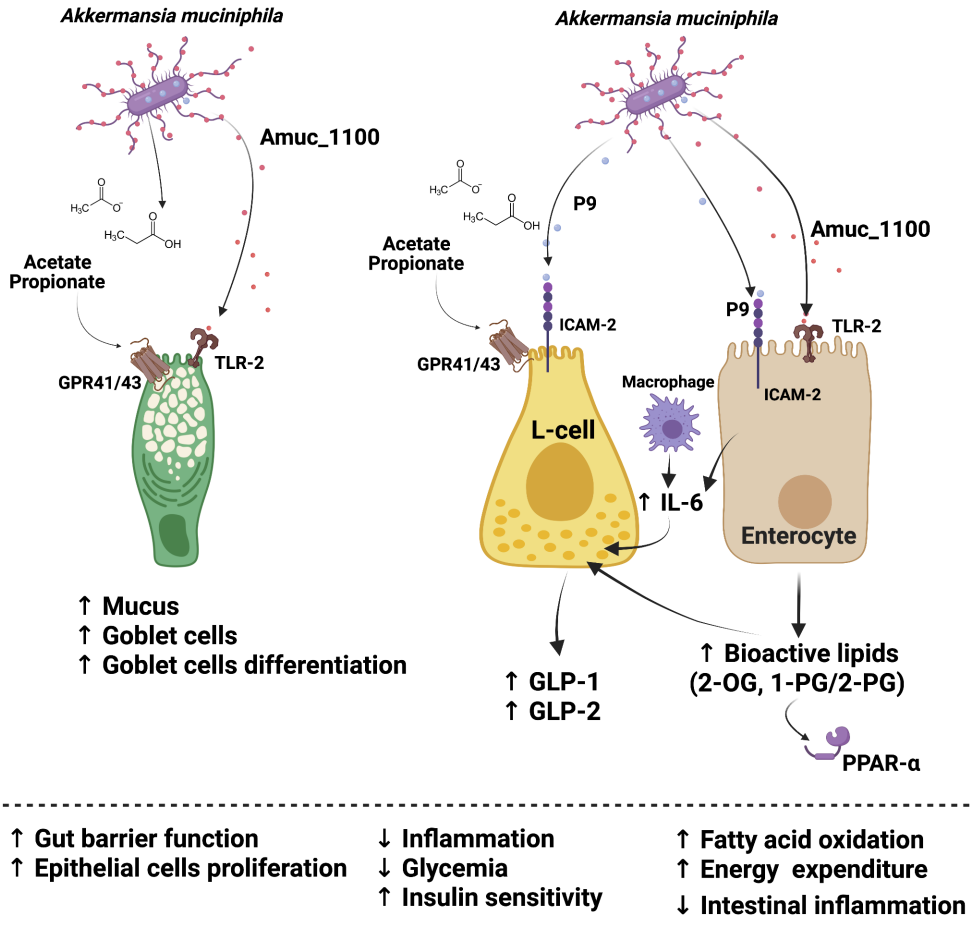


***Figure 8. Molecular mechanisms and modes of actions of A. muciniphila* in the context of metabolic health.**

Both metabolites (SCFAs) potentially secreted proteins (P9) and outer membrane compounds of A. muciniphila (Amuc-1100) are targeting host receptors such as GPR43/41, ICAM-2 or TLR-2. By activating the enteroendocrine L-cells P9 and SCFAs stimulate the secretion of GLP-1, thereby contributing to the regulation of glucose metabolism[45]. By acting on TLR-2 Amuc_1100 is contributing to the regulation of the gut barrier function, antimicrobial peptide production, immune regulation, mucus layer thickness and the regulation of inflammation. A. muciniphila can also stimulate the production

of several bioactive lipids by the host cells and eventually contribute to the activation of the nuclear receptor PPAR-alpha involved in the regulation of mitochondrial activity, thermogenesis, inflammation, lipid, and glucose metabolism. SCFAs: short-chain fatty acids; P9: protein 9; TLR-2: toll like receptor 2; ICAM-2: intercellular adhesion molecule 2; IL-6: interleukin 6; GPR: g protein coupled receptor; GLP-1: glucagon-like peptide-1; PPAR: peroxisome proliferator-activated receptor.

Recently, by using both lipidomic and metabolomic analysis in humans treated with either live or pasteurized *Akkermansia*, our team discovered that *A. muciniphila* Muc^T treatment induces specific modulation of different bioactive lipids that are identified as PPAR-alpha agonist (2-PG and 1-PG) [218]. Strikingly, using untargeted metabolomic analysis they have been able to reconstruct a metabolic pathway pointing towards the activation of fatty acid oxidation via the beta-oxidation and all the metabolites identified were converging towards a higher mitochondrial activity also under the control of PPAR-alpha [219] (Figure 8). It is worth noting that these data have also been confirmed in rodents [204, 220]

Two other studies identified enzymes expressed by *A. muciniphila* Muc^T as potentially beneficial. The first study demonstrated that the aspartic protease Amuc_1434*suppresses LS174T cell viability via TRAIL-mediated apoptosis pathway[221]. The second study demonstrated that a β-acetylaminohexosidase Amuc_2109* expressed by *A. muciniphila* Muc^T protects mice against DSS-induced colitis but no thermal stability data have been reported [222]. Although these findings are relevant, additional tests such as physiological dose and location, effect of pasteurization and direct comparison in different settings between the administration of live *Akkermansia* and pasteurized one should be carried out. The effects of *A. muciniphila* type strain Muc^T were studied for the first time in humans in the "Microbes4U" [203, 223] study which was started in 2015 and published in 2019.

Based on its results, the European Food Safety Authorities decided in September 2021 [224] to recognize the pasteurized form of *A. muciniphila* type strain Muc^T as a novel food. The "Microbes4U©" study was the first, proof-of-concept, exploratory study on humans. It involved a daily ingestion 10^{10} cells/day (in glycerol) of either the alive or the pasteurized form of *A. muciniphila* Muc^T for 3 months with the primary objectives to assess the feasibility, tolerability, and safety profile of that specific strain in a cohort of 40 adults medicine-naïve, newly diagnosed with a metabolic syndrome and a prediabetes state. This intervention allowed for the first time to explore whether this administration translates into potential beneficial metabolic effects in humans. The results showed that *A. muciniphila* Muc^T administration was feasible, safe and well-tolerated in humans suffering from a metabolic syndrome. The study showed that the administration of pasteurized *A. muciniphila* Muc^T successfully prevented the natural deterioration of parameters.

6.4 *Akkermansia muciniphila* and wound healing

Already in 2016, the team of Neish [153] showed a positive correlation between the presence of *Akkermansia* and a favorable wound healing. Between two- and four-days post-mucosal injury, the abundance of anaerobic bacteria, in particular *Akkermansia* spp., increased in early regenerative mucosa (Figure 9). The hypoxic wound environment appeared to be the result of an influx of neutrophils, immune cells that respond to the injury by consuming the oxygen. Alam et al. found that *A. muciniphila* administration plays a major role in wound healing by creating a specific environment and local environmental changes. This state favours a transient consortium of

microbes that can stimulate restitutive signalling pathways to increase both the migration and proliferation of the epithelial cells. The study demonstrated that among the molecular mechanisms, formyl peptide receptor 1 (FPR1) and neutrophilic NADPH oxidase (NOX2) were required to induce the rapid depletion of local oxygen that resulted in the enrichment of anaerobic bacteria (Figure 9). Using mice with a specific deletion of *Fpr1* or *Nox1,* demonstrated that *A. muciniphila* modulate redox-dependent cellular signalling via these two specific actors. These data support previous findings that *A. muciniphila* reinforce the gut barrier function via mechanisms involving the production of mucus and antimicrobial factors in obesity and diabetes [66].

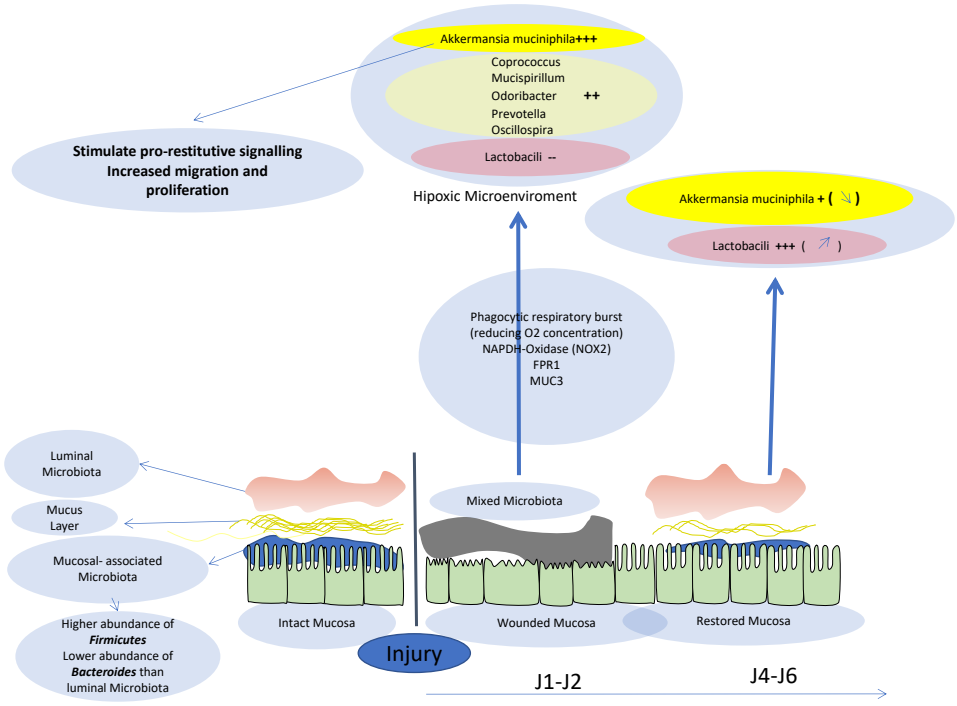


Figure 9: Microbiota shifting as a consequence of the injured mucosa accelerating the healing mechanism

Based on the findings of Alam et al [153], this graph shows the different changes that occur in the microbiota according to the intestinal injury. It shows the important role of the wound-mucosa-associated microbiota in initiating the early healing cascade by stimulating pro-restitutive signalling and increasing cell migration and proliferation.

Several mechanisms by which *A. muciniphila* can exert its beneficial effects and affect host physiology have been analysed. These include improvement of the gut barrier function [210, 225], immunomodulation of the adaptive immune system [226], and activation of anti-inflammatory responses [227].

Box 5: *Akkermansia muciniphila* and wound healing

Akkermansia muciniphila

- increases the mucus production
- increases the restauration of the mucus barrier
- reinforces gut barrier function through immunomodulation by acting on TLR-2 and restoring appropriate tight junction expression

Previously, it has been argued that *A. muciniphila* restores mucin production [228] by being involved in a positive feedback loop, whereby through mucin degradation it also stimulates mucin production (Box 5).

In addition to the physical protection conferred by the mucus layer to prevent pathogens from getting into the body and helping with the wound healing, the biochemical barrier is another important mechanism that helps keep

commensal bacteria at a distance and fend off pathogenic bacteria. Specific antimicrobial peptides (AMPs) [207] such as regenerating islet derived-3 gamma (Reg3γ), phospholipase A2 group II (Pla2g2), Lysozyme C (Lyz1) and angiogenin 1 (Angg1) act as a firewall against certain bacteria by affecting their viability and are involved in intestinal epithelial cell turnover [186]. Furthermore, it has been found that *A muciniphila* reinforces the gut barrier function through immunomodulation by acting on TLR-2 and restoring appropriate tight junction expression. Indeed*, A. muciniphila* has previously been positively correlated with intestinal expression of Reg3γ [66, 186]. Regenerating Reg3γ mRNA expression in enterocytes and Paneth cells is controlled by microorganism-associated molecular patterns through TLRs.

Therefore, given the lack of specific model as well as the use of targeted approaches such as using specific bacterium, the present PhD thesis has been developed to fill several of these gaps.

THESIS AIM

Anastomotic leakage in colorectal surgery is the complication with the most far-reaching consequences related to post operative morbidity as well as the survival rate. Though many improvements in the operative management of colorectal pathologies were implemented over the last decades and a significant reduction in the postoperative morbi- mortality was achieved, this did not translate into a significant reduction of anastomotic fistula rates.

Mechanical bowel preparation associated, sometimes in combination with the administration of local antibiotics, used to be a standard procedure in colorectal surgery. However, this practice has become subject of much debate since the increased implementation of enhanced rehabilitations programs starting 30 years ago in which the goal is to minimize the operative stress on the body by trying to maintain its homeostasis. Already up to 22 recommendations were developed to this end. Though the exact protocol may vary, the most important adaptations relate to the lack of preoperative bowel preparation, the absence of long term fasting, the rapid re-alimentation a few hours after the operation, and the avoidance of opiate derivates administration as post-operative painkiller therapy. These recommendations applied through the “fast-track” concept are in complete contrast to the so-called surgical dogmas, but have led to a significant decrease in the general postoperative complications when compared to classical management and they have also an obvious influence on the intestinal microbiota.

Supporters of the preoperative bowel preparation argue for its utility by putting forward the better results concerning the rate of surgical site infections, but this only applies in cases where an oral antibiotic preparation is associated to the classical mechanical bowel preparation. In fact, the data available so far does not allow to determine clearly if the combination of administration of the mechanical bowel preparation with the use of the oral antibiotics is decreasing the rate of surgical site infections or if this is due only to the use of the latter. Occurrence of surgical site infections is

associated with an increased length of stay at the hospital as well as with higher therapy costs and the later occurrence of surgical site hernia, but does generally not significantly increase the post operative mortality.

Today, the scientific community has not yet been able to reach a consensus on whether or not to use mechanical bowel preparation prior to colorectal surgery. This is partly because of the realization that it does not significantly influence the occurrence of an anastomotic leakage.

While the question ***"to prep or not to prep?"*** is still very much at the center of the debate, new insights coming from preclinical studies may offer the opportunity to completely revise our concept of intestinal preparation: perhaps it's time to evolve towards a more **targeted preparation**.

Indeed, since the recent "*rediscovery*" and increased understanding of the role of the intestinal microbiota, many experimental preclinical data suggest the potential involvement of the local microbiota in the healing of a colorectal anastomosis. Classical preparation protocols have a high risk of depleting and/or altering the local microbiota and, in consequence, result in the elimination of potentially beneficial bacteria. We know for example that while preparation with PEG solutions does not decrease the total number of bacteria, it does reduce their diversity, with deleterious bacteria being more persistent. In addition, it is well described that while the administration of oral antibiotics almost sterilizes the intestinal mucosa momentarily, bacterial recolonization occurs after very rapidly with deleterious bacteria expanding more quickly than they would in a well-mixed population.

The whole concept of a more targeted preparation procedure therefore relies on the possibility to locally increase the number of "good" bacteria while avoiding to increase harmful bacteria.

To be able to define what a potential targeted preparation needs to contain in order to achieve a significant improvement in the post-operative morbidity

and wound healing, mechanistic studies, aiming at understanding how the local microbiota influences these aspects, are crucial.

As presented in the introduction of this thesis, the process of intestinal wound healing presents different phases, with very complex cross-talks between the healing tissue and the local microbiota. However, current knowledge on these complex interactions is still scarce.

In this thesis we aimed at identifying a **beneficial bacterium** which might be used pre- or perioperatively in order to decrease the occurrence of anastomotic leakage. To do this, we need a reliable model, allowing to test the influence of the local microbiota on the intestinal wound healing. After studying and testing the existing animal intestinal leakage models proposed in the literature, we concluded that the existing models have a lot of constraints and are not suitable for the purpose of this research, namely, to be able to easily modify the local microbiota and study the local conditions.

The **first aim of my thesis** was therefore to **develop a new animal model** to study different conditions associated with the wound healing that would meet the following requirements: be reproducible, easy to perform, cause minimal pain for animals with rapid recovery and have less dropouts than the classical model (mostly due to due to intestinal occlusions) than the classical models.

We would then use this new model to achieve our second objective of the thesis, namely to modulate the microbiota in such a way that we could improve the wound healing and morbidity.

For this purpose, we decided to use a bacterium called ***Akkermansia muciniphila***, based on previous beneficial properties attributed to this bacterium: first it has been shown that *A. muciniphila* can increase intestinal

mucus production, a key component of the gut barrier; second it has also been shown, that *A. muciniphila* can reinforce the gut barrier by acting on TLR2 and restoring appropriate tight junction expression.

In a later stage, we would try to **decipher, at least partially, the potential mechanism** of action of *A. muciniphila* in wound healing progression.

Finally, we built on an international collaboration reuniting a group of researchers from Finland, Sweden and the Netherlands, in order to realize an exploratory **proof-of-concept study,** to analyze whether the local colonic instillation of *A. muciniphila* is safe **in human volunteers** and whether it is sufficient to induce some local transcriptomic effects, while also assessing the level of *A. muciniphila* pre and post instillation in the feces and in the mucus layer.

In summary, the overall purpose of our project, of which this PhD thesis is a part, is to develop a potential targeted intestinal preparation in colorectal surgery with the purpose to significantly reduce the rate of anastomotic leakage.

RESULTS AND DISCUSSION

Chapter 1: The colonoscopic leakage model: a new model to study the intestinal wound healing at molecular level

Adapted from [229]

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The colonoscopic leakage model: a new model to study the intestinal wound healing at molecular level

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Message

We describe a novel, fast-track, innovative intestinal wound healing model called “colonoscopic leakage model” which will enable to investigate more easily the different conditions of wound healing at local level and to decipher the different mechanisms involved. This model has a number of advantages: it is time effective, reproductible, easy to perform and with less confounding factors than other current existent models. This innovative model will be useful to study the underlying molecular mechanisms of interaction between the local microenvironment and the intestinal wound healing. Furthermore, it represents a potential high-throughput model for the validation of local use of different pharmacological and/or therapeutic tools.

In more detail

Despite significant progress in the medical field, the mechanisms of healing of a wound in the GI tract are still not completely identified, which limits the options for positive interventions at local level to support the healing processes.

Even though many influencing factors have been studied, it is very challenging to acquire knowledge of the local patho-physiology and to study the impact of different pharmacological and microbiological conditions at molecular level. To overcome these difficulties, the use of experimental models that help understanding the local conditions have become indispensable.

The models proposed in the literature to study the mechanisms of intestinal wound healing are mainly anastomotic leakage models. Amongst them, the one proposed by Pommergaard [230] is most commonly used technique (**Online supplementary video 1** [231]). This rodent model is based on the variation of the number of intestinal anastomotic sutures after segmental colonic resection (5 versus 8 stitches) in order to achieve a probability of 50% of anastomotic leakage (**figure 1A and Online supplementary video 2 to 4** [232]) and peritonitis in the anastomotic leakage group.

This model, that we adapted to our laboratory settings in order to achieve the same outcome, is feasible and reproductible; however, during our research we identified a number of significant shortcomings that limits the use of this model for potential transversal clinical approaches.

The most important problem is the occurrence of occlusions, mostly in the anastomotic leakage group, with the intestinal transit being seriously affected

(**figure 1B and C**). This makes it difficult to perform local interventions and study changes at molecular level. We tested several optimization conditions but without significant improvement. For example, after controlling the tightness of the anastomosis for air and liquid, we further checked for the absence of stenosis, by passing a rectal canula through the anastomosis or even through colonoscopy done at the end of the surgical procedure. We also tested the possibility to use an alternative solid diet or even a liquid diet. To improve general welfare and recovery, we made several modifications in the postoperative care protocol, such as keeping the animals in heated cages and adapting the use of analgesics. The local interventions were either done by gavage or intrarectal application. However, none of these conditions significantly changed the frequency of occlusion occurrences significantly.

Another limitation of this model is that it is extremely time consuming, making it difficult to reach sufficient numbers of animals needed to study different conditions in a statistically relevant manner (**figure 1B and C**). Finally, this model also proved to be very invasive as the subject lost significant weight and recovery was very slow. In fact, the mice did not return to their initial body weights within a time window of 7 days (**figure 1D**).

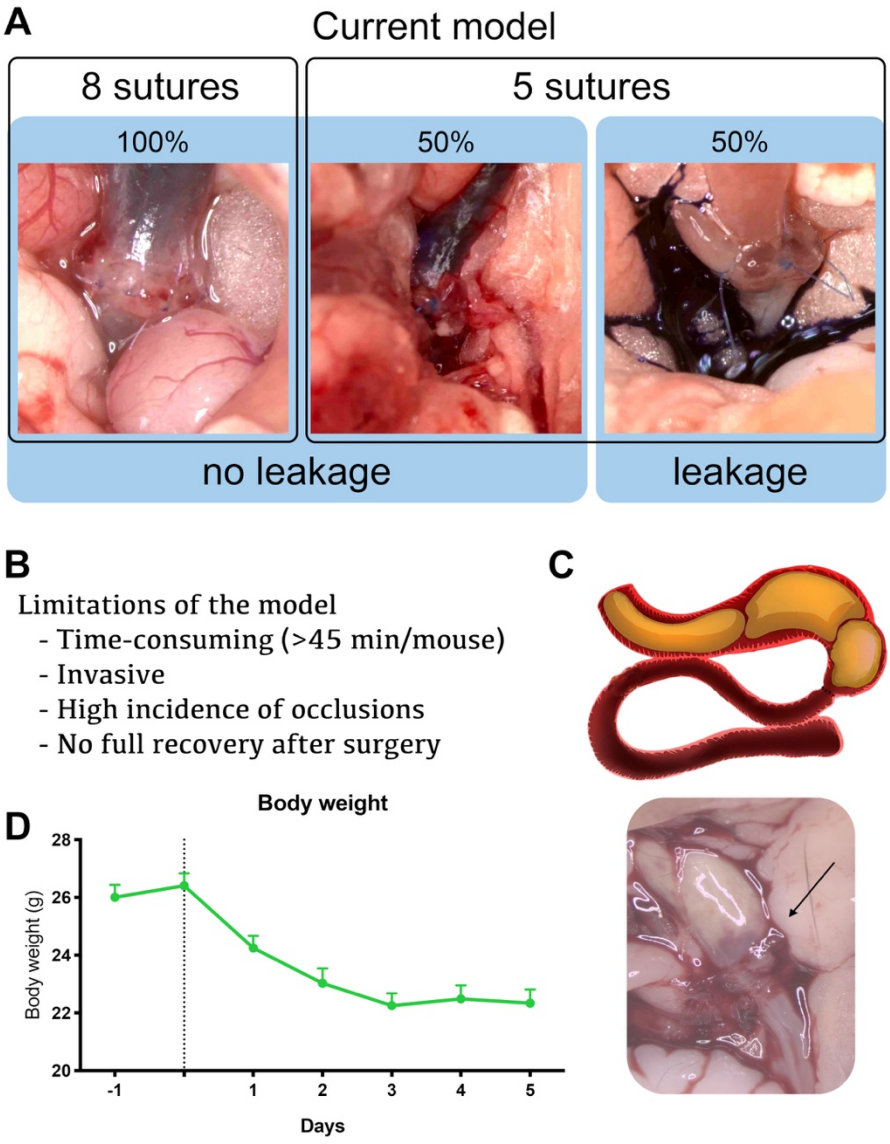

Figure 1: Standardisation of the anastomotic leakage model

*(**A**) The anastomotic leakage model is feasible with a percentage of anastomotic leakage and peritonitis of 40-50%. The tightness and the absence of stenosis is controlled postoperatively. (**B**) However, this model has some important shortcoming if the purpose is to study the local conditions. (**C**) The most important one is the occurrence of intestinal*

*obstruction. (**D**) In addition, there is a general body weight drop of over 15% with little to no recovery after 5 days.*

In order to address these weaknesses, we set up a new intestinal wound healing model, using a colonoscopy and a grasper that we called "***Colonoscopic Leakage Model***" (**figure 2**). As described further below, this model is easy to perform and has numerous benefits compared to the current anastomosis leakage model concerning the study of the local conditions and for testing pharmacological manipulation.

Similar to the anastomotic leakage rodent model described by Pommergaard, we are creating a controlled transmural perforation in a normal vascularized colon. However, in our method a transmural biopsy is performed during a colonoscopy with the aid of a grasper passed through the working channel of the colonoscopic system (**Online supplementary video 5** [233]). Working under direct visual control, we are able to precisely determine the site of the perforation of the colonic wall (**figure 2A, B and C**). Beside this visual confirmation (**Online supplementary video 6** [234]), successful perforation is also objectivated by the immediate occurrence of a pneumoperitoneum and instantaneous inflation of the whole abdominal cavity. The deflation of the peritoneal cavity is obtained by intraperitoneal introduction of a needle for a few seconds (**Online supplementary material and methods**). Performing the whole procedure takes less than 10 minutes (i.e., 6 times faster than the anastomosis model). Additional advantages include a fast recovery of the mice, with mice displaying normal behaviour as soon as the anaesthetics wear off, and a low use of pain killers without the necessity of using opiates.

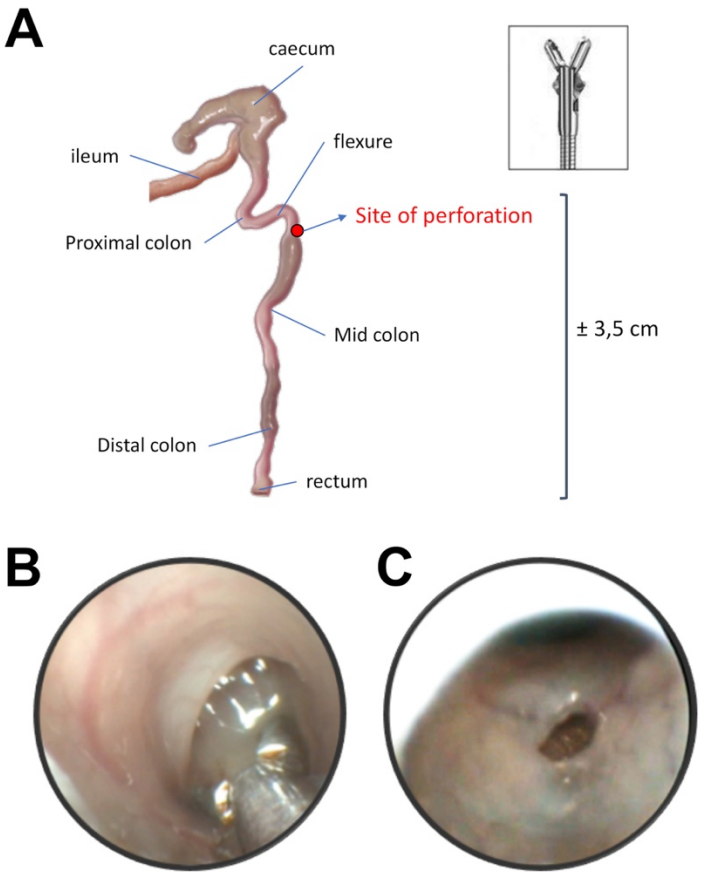

Figure 2: Standardisation of the colonoscopic Leakage Model

*(**A**) The transmural biopsy is at 3,5 cm from the anal verge, lateral to the mesocolon, (**B**) done with 3 Fr-biopsy forceps by opening the jaws completely. The local conditions are kept similar, with a working pressure set to 3 mmHg. (**C**) Representative perforation visualized by endoscopy.*

Weight loss is also very limited, and mice reach their pre-operative body weights in less than 5 days (**figure 3A**). Importantly, we did not observe any

signs of intestinal occlusion, therefore ensuring a normal bowel movement after the procedure.

After 7 days of follow-up, our model resulted in 40-50% of incidence of peritonitis which is perfectly in line with what was obtained using the anastomotic leakage model (**figure 3B**). These favourable conditions allow us to study the local/mucosal conditions and possibly test different nutritional approaches.

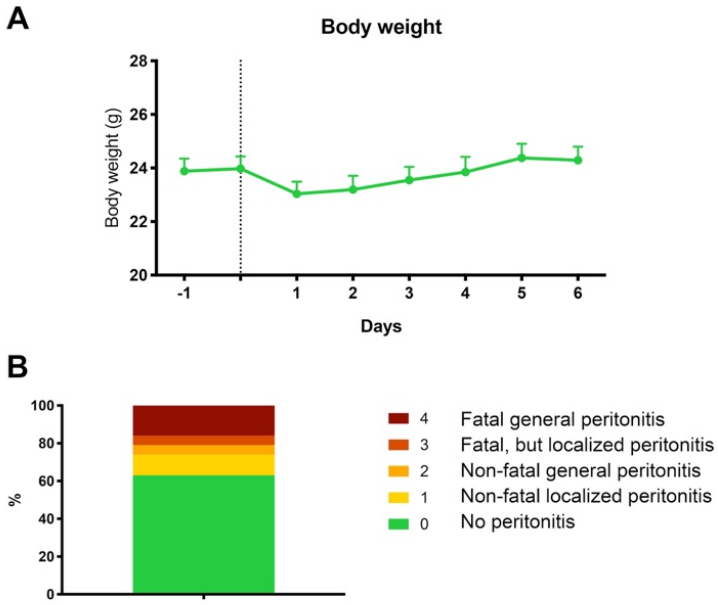

Figure 3: Colonoscopic leakage model: Body weight evolution and peritonitis score at the predefined endpoint

*(**A**) Weight loss is very limited, and mice reach their pre-operative body weights in less than 5 days. (**B**) The percentage of peritonitis is of 40-50 % at 7 days post-intervention. The subject's mortality before the selected endpoint (i.e. 7 days) is between 10 -19%*

A detailed description of this cicatrisation model is provided below, with video documentation of the colonoscopy procedure from inside the bowel (**Online supplementary video 5 and 6**) as well as an external overview (**Online supplementary material and methods**).

To characterize the levels of inflammatory response within our test subjects, we also developed a peritonitis severity score (**figure 3B**), ranging from 0 (no peritonitis) to 4 (peritonitis affecting the whole abdominal cavity and that led to death before the end of the 7-day period) (**Online supplementary material and methods**).

Using this model, changes at a local level can be studied using complementary techniques: colonic sites of interest can be isolated and used to measure molecular biology markers (e.g. via qPCR or Western blot), or the area of perforation can be prepared for histological analysis (**figure 4**). Such microscopical evaluation grants a deep morphological characterization when using different staining protocols (i.e. haematoxylin and eosin for general morphology, Alcian Blue for mucus layer, immunohistochemistry). This approach also has the advantage that different sections of the colon can be compared and that combining several serial sections into a 2D reconstruction can provide a broad overview of the healing process around and at the perforation site (**figure 4**).

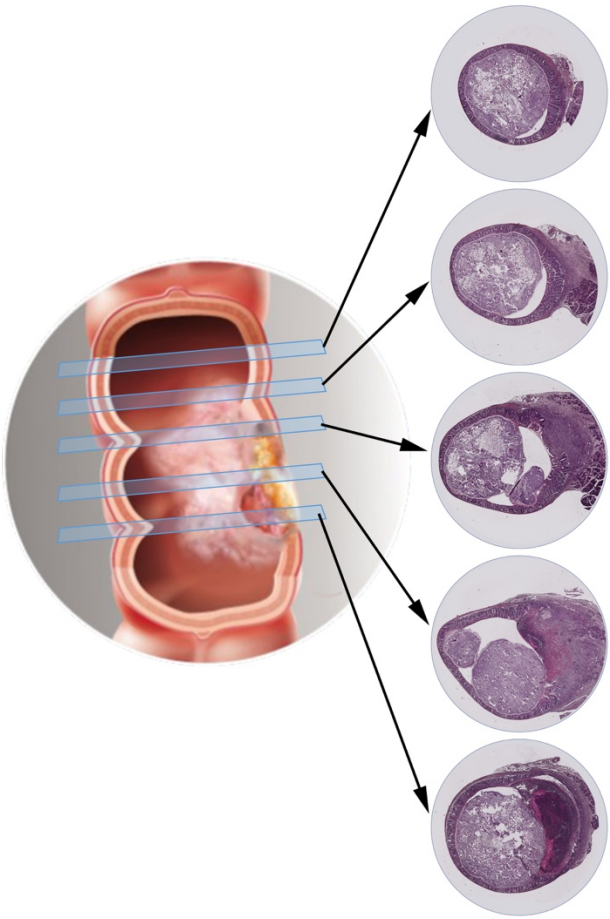

Figure 4: Histological view of the wound healing area using a serial slicing method.

This procedure allows to continuously see the entire wound healing zone, starting from the intact part of the colon and then approaching the site of perforation. Series of histological analysis surrounding the perforation allows to investigate the evolution of the wound healing process: from normal mucosal layer, to a progressive thickening of the mucosal and muscular layer and an accumulation of infiltrating cells. A clear disruption of the mucosal layer when arriving at the border of the lesion with abundant growth of

fibroblasts and capillaries into the gap replacing the muscular layer and massive accumulation of infiltrating cells when advancing in the centre of the lesion.

Material and Methods

Animals

All mouse experiments were approved by the Ethical Committee for Animal Care of the Health Sector of the Université catholique de Louvain, under the supervision of Prof. JP Dehoux under number 2018/UCL/MD/013 and were performed in accordance with the guidelines of the Local Ethics Committee and in accordance with the Belgian Law of May 29, 2013, regarding the protection of laboratory animals (agreement number LA1230314).

For all experiments, male C57BL/6J mice were purchased from Janvier (Le Genest-Saint-Isle, France) and were housed in pairs under strict specific and opportunistic pathogen free (SOPF) conditions and in controlled environment (room temperature of 22 ± 2 °C, 12 hour daylight cycle, lights off at 6 pm) with free access to food and water.

In all our procedures, we abstained from using antibiotics either pre- or post-intervention.

Adapted Anastomotic Leakage Model

More and more studies suggest that the mouse is the most suitable animal to realize an anastomotic leakage model [235]. We decided to implement the anastomotic leakage model proposed by Pommergaard [230, 236] in our facility. Briefly, this murine model of colonic anastomosis after short resection, aims to obtain a postoperative probability of 50% of anastomotic leakage and peritonitis. This is achieved by varying the number of surgical sutures used to perform the anastomosis, with either 4 separate points for the "peritonitis-group" or an 8 separate point colonic anastomosis for the "control-group" in which no intra-abdominal infections are expected. The only difference between the two groups are the gaps between two adjacent stiches or, in other words, the size of the five transmural wound which are present between each two adjoining stiches. The resections/ anastomosis is done in the left colon, below the left colic angle. The colonic vascularisation is preserved, and the anastomosis is done tensionless.

In our laboratory, we made use of a surgical trinocular with 20-40 X magnification with the possibility of filming through the extra optic (Nikon SMZ800N). Microsurgical instruments (needle holder, scissors and forceps) were purchased from FST (Fine Science Tools, Heidelberg, Germany) and special attention was paid to choose an adequate atraumatic suture tying forceps in order to avoid involuntary perforations when handling the intestines.

Our procedure went as follow:

Anaesthesia was performed using isoflurane gas (Forene, Abbott, Queenborough, Kent, England), in accordance with welfare criteria [237]. We used a dose of 3% for induction and of 2,5 % during the intervention. Mice

were placed on a heated plate to ensure the body temperature did not drop too low.

After gently pinching the hind paw and confirming the loss of reflex, the abdomen was then disinfected and a midline skin incision of 2 cm was made with scissors (better than using scalpel) in the middle third of the abdomen, after which, the muscular abdominal wall was lifted away from the intestine in order not to injure any intra-abdominal organs and cut at the midline, between the two rectus abdominis muscle, in a complete avascular plane.

An intraperitoneal injection of buprenorphine 0.3mg /ml diluted 100 times (Buprecare; Animalcare Limited, UK) with the µl-equivalent of 6 times the weight of the mouse was administered.

The caecum and rectum were used as reference points to locate the site intervention, about one centimetre caudal of the left colic flexure. The resection segment should not be larger than 2-3 mm, in order not to put too much tension on the future anastomosis. Particular attention should be paid on the mesocolic vascularisation and the resection segment should be chosen in a zone between two vascular branches, in order note to induce any bleeding or impair the vascularisation of the two colon edges.

It is also important to avoid manipulating the intraabdominal structure with graspers when searching for the site of intervention, as this could cause unwanted damage. It is preferable to use sterile nasal cotton plugs for this purpose. Also, the intraabdominal organs should not be taken out of the abdominal cavity in order to create working space but kept in the abdominal cavity which should be moistened at regular intervals using saline solution.

The segmental resection of the colon is performed using scissors, taking care not to damage any blood vessels. The end-to-end colo-colic anastomosis is done in *Nardi* technique using 8-0 Vicryl® interrupted inverted sutures. The stiches need to be placed at equal distances,

especially if doing 5-point anastomoses. Applying first stich at the mesenterial level simplifies the rest of the procedure. If doing an 8-point anastomosis, the second stich should be placed at the anti-mesenterial level, followed by three stiches at equal distances on both anterior and posterior faces.

The tightness of the anastomosis can be verified directly postoperatively, before closing the laparotomy wound, either by covering the anastomosis with saline solution and insufflating air rectally, or by using a solution of Methylene blue 1% (Neopharma, Aschau, Germany) that was introduced via a flexible rectal probe. The latter was our method of choice as it allowed better visual control and was easier and faster (see online Videos showing the presence of the absence of leakage).
Beside the tightness, we checked also for lack of stenosis in the anastomotic region. This can be done either by using a colonoscope with a 3mm endoscopic sheath or by passage of a 20 Ga x 30 mm disposable plastic feeding tubes through the anastomosis.

After verifying the tightness and lack of stenosis, the abdominal wall is closed using a 4-0 Vicryl® running sutures, followed by closure of the skin using a running 5-0 Byosine™ suture. At the end of the intervention, 150 µl of Ketofen 10%, 100mg/ml diluted 100 times (Ketofen; Ceva Sante Animale, France) was administered Intraperitoneally.
The average intervention time was 45 minutes per mouse. The first two hours after surgery, mice were placed in a climate chamber set at 25°C to improve recovery. Mice that underwent the procedure were observed twice a day and their well-being was assessed using the wellness score proposed by Komen [238]. They were weighed once a day.

Painkillers were administrated for four days after intervention. Postintervention analgesia was multimodal, using a combination of paracetamol and NSAIDs (Ketoprophene; Merial, Belgium) administered subcutaneously, for 48 hours postoperatively, followed by Paracetamol alone, administered orally (in the drinking water), for another 48 hours. Under these conditions, postoperative pain was estimated to be moderate.

After 7 days, mice were anesthetized with isoflurane. Evaluation of the severity of the peritonitis using our Peritonitis Score was done by two different investigators which were blinded to the mouse groups. The concordance between the two evaluators was 100%.

Evaluation of the Pommergaard-Adapted Anastomotic Leakage Model

In our hands, the 50 % probability of anastomotic leakage and peritonitis was obtained when performing a 5 separate point colonic anastomosis, as four suture points almost always resulted in leakage.

As also remarked by Pommergaard, one of the most important shortcomings of this model is the occurrence of large bowel obstruction. This is caused on the one hand by the surgical traumatism at the level of the anastomosis, and on the other hand by the peri- and postoperative analgesic regimen used during the recovery period, as the administered mixture is known to impact on bowel movement and transit time [239]. Neither of these causes can be eliminated, as surgical trauma and reduced flexibility is inherent to the procedure and the perioperative administration of opiates is mandatory for the well-being of the animals.

Contrary to rodent bariatric surgery models, in which provision of liquid nutrition during the perioperative period is standard practice [240], supplying liquid diet (Liquid Diet, AIN-76, Bio-Serv, Flemington, NJ) did not minimizes the risk of postoperative intestinal stasis and obstruction in our experiments. We did not observe an effect of the liquid diet on body weight loss, but coagulation of the liquid diet, even within specially adapted delivery systems, made it very impractical, reason why we chose to continue to use our standard diet throughout the rest of the experiment.

After performing different pilot studies in order to fine-tune the experimental set-up and achieve similar results as those described by Pommergaard, we started an experiment in which 11-weeks old male C57BL/6J mice were randomly assigned to one of three experimental groups: two groups underwent anastomosis with either eight (n=8) or five (n=7) separate sutures, the third group (n=6) had a sham laparotomy without intestinal resection/anastomosis.

In the sham laparotomy group, we did not observe any peritonitis or weight loss. The mice displayed no signs of discomfort or sufferance and there was nothing to indicate their bowl movement was affected. In the anastomotic groups we had a general body weight drop of over 15% with little to no recovery after 5 days.
We obtained 53% peritonitis in the anastomotic leakage group with 5-point anastomosis and 12,5% peritonitis in the control group with 8-point anastomosis, indicating this model is feasible and reproductible.
However, we also found sub-occlusion in 53% of the animals (excluding the sham group) and even observed complete bowel obstruction with massive dilatation of the small intestine in some animals (20%). The occlusions were most prevalent in in the Anastomotic Leakage group (63% in animals with 5

sutures) and the rest in the control group (8 sutures) and were not always corelated with the appearance of a local or general peritonitis.

These outcomes were incompatible with our main experimental purpose which is to study the intestinal wound healing at molecular level while being able to evaluate the impact of different local manipulations.
Moreover, this model is very time-consuming, making it difficult to obtain sufficient animals per experimental group, especially since the drop-out rates are high due to the high occurrence of intestinal obstruction.

Taking all these elements into account, we set out to develop a better, more reliable and more efficient model of intestinal wound healing in the mouse.

We noticed that the gap between two adjacent stiches in the case of a 5-point anastomosis is more or less equal to the opening of our 3Fr. colonoscopical grasper. With this in mind, we imagined a model of endoscopic transmural perforation, in which a single puncture was created at a precise location during a simple colonoscopical examination. We termed this the "*colonoscopic leakage model*". This model was designed to resemble clinical reality of small intestinal perforation as is the case with perforated diverticulitis and iatrogenic colonoscopic perforations.

Colonoscopic leakage model

This procedure necessitates a white light colonoscopic system designed for use in rodents, with a monitor for easy visualization of the colon throughout the procedure (for references of the system, see below). We used a system combining a high-resolution camera with a rigid

examination sheath that enables local manipulation through an endoscopic sheath working channel.

The feasibility of practicing rigid colonoscopies in mice has been described in 2006 by Becker and al. [241] and it has since then been used mostly to asses colonic dysplasia development [242] or the inflammatory conditions in colitis [243], though never in the context of transmural wound healing.

One of the advantages of using this technique, is that the mice do not need a preoperative bowel preparation, nor do they need to be fasted before the intervention. In fact, contrary to observations on rats [244], our pilot experiments showed that an overnight or even a 24-hours fasting is not sufficient to completely empty the intestines and provide adequate bowel preparation. However, in order to perform colonoscopical examinations in optimal conditions, we suggest flushing the colonic lumen to remove any faeces that may hamper visibility or prevent access to the site of interest. To do so, we suggest using flexible soft-tip feeding needles to administer a trans-anal enema with 1 ml of saline solution 10-15 minutes before the intervention. This has proven efficient in ensuring a colonoscopical-clear view without residual gut content up to the left colonic angle. Of note, performing this enema too early (one or two hours before the procedure) was not effective.

The correct adjustment of the CO_2 flow is of crucial importance. A CO_2 flow rate of 0,4 L/min and a working pressure set to 3 mmHg was optimal to obtain sufficient insufflation of the colon without creating an overpressure, that may in certain cases lead to suffocation of the animals. Indeed, we observed that augmenting the pressure to 6 mmHg resulted in an important incidence of peri-interventional fatalities caused by a massive dilatation of the stomach. Similar problems were encountered when trying to improve visibility by

flushing the intestines during the intervention with volumes of saline solution larger than 1 ml.

The procedure includes the following steps:

Anaesthesia was performed as described above (3% induction and of 2,5 % maintenance dose). There is no need to place mice on a heated on a hot plate as this is a fast and non-invasive procedure. The examination sheath should be introduced gently and about 1 cm from the anal verge. After fitting optical fibre and the camera, the examination sheath can be guided through the colon until just before the left colic flexure, i.e. 3,5 cm ab anno and lateral to the mesocolon. We do not recommend the use of any lubricant for the introduction of the colonoscope, as this may blur the image if smeared on the optic fibres. Marking the sheath with a reference point, makes it easier to standardize the location.

3Fr-biopsy forceps can then be introduced through the endoscopic sheath working channel.

The transmural wounding is performed by opening the forceps at the desired angle, pressing it against the colonic wall, grasping and pulling while twisting. The biopsy obtained this way can be collected and snap-frozen for further analysis, if needed.

Successful perforation is immediately objectivated by the manifestation of a pneumoperitoneum with instantaneous inflation of the abdominal cavity. If not maintained for too long time, this has no impact on the wellbeing of the mouse. The deflation of the peritoneal cavity is achieved by intraperitoneal introduction of a needle (19G) for a few seconds to allow excess gases to escape.

Mice can then be returned to their habitual environment, there is no need to keep them in a climate chamber.

Because this is a fast procedure, maximum efficiency is achieved when performed by three persons: one is in charge of managing the animals (weighing and flushing the animals prior to the procedure, timing initial anaesthesia and returning animals to their cages), one is doing the colonoscopy and maintains the correct positioning, while a third person manipulating the grasper, performs the actual perforation and collects the biopsy.

The entire procedure takes less than 10 minutes (i.e., 6 times faster than the anastomosis model). Additional advantages include very low peri-interventional mortality and fast recovery of animals, with mice displaying normal behaviour as soon as the anaesthetics wear off, eliminating the necessity of using high doses of opiates. Weight loss is also very limited, and mice return to their pre-operative body weights in less than 5 days. Importantly, we did not observe any signs of intestinal occlusion, indicating the procedure does not impair normal bowel movement.

If all the precautions described above are considered, this method generates a percentage of peritonitis of 40-50 % during the first 7 days post-intervention. This is similar to the results obtained in the anastomotic leakage model, but without all the aforementioned shortcomings.

The mortality rate before the selected endpoint (i.e. 7 days) is between 10 - 19%, which is much lower than that of the anastomotic leakage model. Moreover, all of these were due to either a localized or generalized peritonitis.
Because we observed distinct gradations of inflammatory response within the animals, we developed a proposal for the use of a peritonitis score, which defines five end-point categories groups:

0) mice in which no peritonitis was observed,
1) mice with a peritonitis that was localized only to the site of perforation and that did not result in death,
2) Mice with a generalized peritonitis affecting the whole abdominal cavity, but that did not result in death,
3) mice that died before the end of the 7-day period that had only a localized peritonitis and 4), mice that did and displayed clear signs of a generalized peritonitis.
All categories were given a specific score to allow a more sensitive appreciation of the outcome and a fast evaluation of comparative experimental designs. The reproducibility of this scoring was confirmed independently by two different, blinded examiners without any difference in the evaluation.

Score	**Groups**
4	Fatal general peritonitis
3	Fatal, but localized peritonitis
2	Non-fatal general peritonitis
1	Non-fatal localized peritonitis
0	No peritonitis

At necropsy, blood and tissues can be sampled as usual. In addition, the site of perforation can be identified and prepared for protein/RNA extraction or for histological analysis. We recommend using a surgical trinocular.

Equipment and Materials:

1. **Colonoscopic tower**: Telepack X Video Unit/ portable all-in-one system from Karl Storz, Tuttlingen, Germany (with possibility of digital recording included)
2. **Insufflator**: Karl Storz Electronic human CO2 Endoflator 26430520 set up with a CO_2 flow rate set to 0,4 L/min and a working pressure set to 3 mmHg
3. **Optics**: HOPKINS rigid Telescope, 1.9 mm, Length 10 cm
4. **Camera:** Karl Storz TELECAM One-Chip Camera Head, PAL, F = 25-50mm
5. **Light Cable**: Fiber Optic Light Cable, Ø2.5mm Karl-Storz
6. **Endoscopic Sheath:** either a protect and examination sheath 7 Fr. Length 8,5 cm without presence of a working channel or an examination sheath 9 Fr. Length 7 cm with presence of a working port.
7. **Grasper:** Biopsie Forceps3 Fr.

Comments and Conclusions

Intestinal wound healing is influenced by a number of factors. While, microbiota-independent environmental factors have already been well studied [16], more and more studies suggest that the local intestinal microenvironment, shaped by the resident microbiota, significantly influences the cicatrisation progress of an intestinal wound [8]. Little research has been done so far in this respect, compared to the potential benefits that this could bring. Indeed, being able to study the local conditions of a wound during healing, including the evolutions in host-microbial relations, has the

potential to help develop specific local interventions that have beneficial effect on recovery.

The current anastomotic leakage model described in literature, although feasible and reproductible, was not satisfying for our research purposes as it presented many unacceptable limitations. The newly developed colonoscopic leakage model is more suitable to study intestinal wound healing at the local level. The model was optimized and validated and can be used to study not only the different wound healing stages but can also be implemented to evaluate modifications of the local conditions after testing different new pharmacological tools, including microbiota-related approaches.

As a consequence, this model has a clear potential for clinical use. It might be used to study the local intestinal wound healing, for example after iatrogenic colonic perforation due to extended polypectomy (i.e. Endoscopic submucosal dissection), or the local cicatrisation mechanisms following a surgical colonic anastomosis. It may offer to the clinicians an easy model to study intestinal cicatrisation under a variety of different conditions such as during treatment with different pharmacological compounds, during nutritional interventions, after modification of the microbiota (e.g., faecal material transplantation), but also in numerous pathologies that impact of the local microenvironment, like autoimmune diseases.

Acknowledgments

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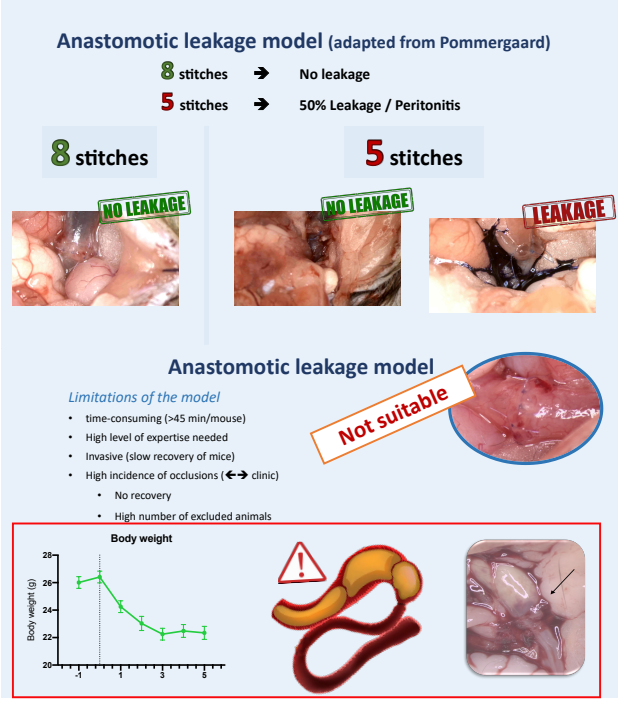
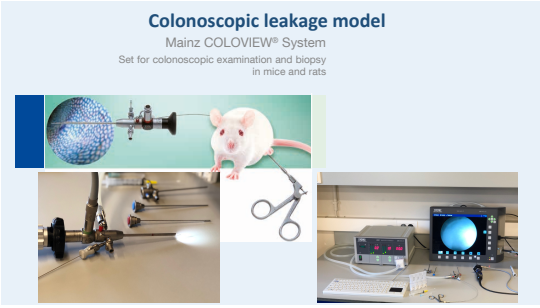
Authors Contributions RB, MHV and PDC study concept and design of the study. RB and MVH performed the experiments. PDC supervised the experiments. RB, MVH and PDC have analysed experiments. RB, MHV and PDC have written the manuscript. PDC provided funding. All authors provided intellectual inputs. All authors critically revised and approved the manuscript. RB and MVH contributed equally.

Competing Interests statement. The authors have no competing interest regarding the content of this study.

Legends supplementary figure and video



Supplementary Figure 5: Schematic description of the anastomotic leakage model and its constraint for our purpose



Supplementary Figure 6: The colonoscopic tower used for the colonoscopic leakage model

Online supplementary videos are posted on the website of the journal *GUT*

Online supplementary video 1: Anastomosis procedure

Visualisation of the intestinal segmental colonic resection and anastomotic sutures

Online supplementary video 2: Absence of leakage after surgical sutures with 8 stiches to perform the anastomosis

Visualisation of the intestinal anastomotic sutures composed of 8 stiches and the absence of anastomotic leakage when flushing the colon with methylene blue.

Online supplementary video 3: Absence of leakage after surgical sutures with 5 stiches to perform the anastomosis

Visualisation of the intestinal anastomotic sutures composed of 5 stiches and the absence of anastomotic leakage when flushing the colon with methylene blue.

Online supplementary video 4: Anastomotic leakage after surgical sutures with 5 stiches to perform the anastomosis

Visualisation of the intestinal anastomotic sutures composed of 5 stiches and anastomotic leakage after flushing the colon with methylene blue.

Online supplementary video 5: Transmural biopsy

A transmural biopsy is performed during a colonoscopy with the aid of a grasper passed through the working channel of the colonoscopy system

Online supplementary video 6: Perforation of the colonic wall

Direct visual control of the perforated colonic wall during colonoscopy

Chapter 2: *Akkermansia muciniphila* reduces peritonitis and improves intestinal tissue wound healing after a colonic transmural defect by a Myd88-dependent mechanism

Adapted from [245]

cells

MDPI

Article

Akkermansia muciniphila Reduces Peritonitis and Improves Intestinal Tissue Wound Healing after a Colonic Transmural Defect by a MyD88-Dependent Mechanism

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Short Title: ***Akkermansia* reduces peritonitis and colon leakage**

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Abstract

Anastomotic leakage is a major complication following colorectal surgery leading to peritonitis, complications, and mortality. *Akkermansia muciniphila* has shown beneficial effects on the gut barrier function. Whether *A. muciniphila* reduces peritonitis and mortality during colonic leakage is unknown. Whether *A. muciniphila* can directly modulate the expression of genes in the colonic mucosa in humans has never been studied. We investigated the effects of a pretreatment (14 days) with live *A. muciniphila* prior to surgical colonic perforation on peritonitis, mortality, and wound healing. We used mice with an inducible intestinal-epithelial-cell-specific deletion of MyD88 (IEC-MyD88 KO) to investigate the role of the innate immune system in this context. In a proof-of-concept pilot study, healthy humans were exposed to *A. muciniphila* for 2 h and colonic biopsies taken before and after colonic instillation for transcriptomic analysis. Seven days after colonic perforation, *A. muciniphila*-treated mice had significantly lower mortality and severity of peritonitis. This effect was associated with significant improvements of wound histological healing scores, higher production of IL22, but no changes in the mucus layer thickness or genes involved in cell renewal, proliferation, or differentiation. All these effects were abolished in IEC-MyD88 KO mice. Finally, human subjects exposed to A. muciniphila exhibited an increased level of the bacterium at the mucus level 2 h after instillation and significant changes in the ex-pression of different genes involved in the regulation of cell cycling, gene transcription, immunity, and inflammation in their colonic mucosa. *A. muciniphila* improves wound healing during transmural colonic wall defect through mechanisms possibly involving IL22 signaling and re-quiring MyD88 in the intestinal cells. In healthy humans, colonic administration of *A. muciniphila* is well tolerated and changes the expression of genes involved in the immune pathways.

Keywords: *Akkermansia muciniphila*; wound healing; colonic leakage; peritonitis; Myd88

Introduction

Anastomotic leakage remains a major and potentially life-threatening complication following colorectal surgery. Despite numerous technical surgical advancements and the development of perioperative enhanced recovery methods in the last decades [6, 7], the percentage of colonic anastomotic leakages did not ameliorate significantly. Literature shows that the prevalence of anastomotic leakage ranges from 1 to 19 %, depending on the anatomical site, and that, in addition to the economic implications, this has direct impact on survival, quality of life, and cancer recurrence [8, 9]. The mortality rates after the appearance of an anastomotic leakage vary in the literature typically from 15 to 33% [10].

Despite growing knowledge on the matter, it is still difficult to accurately predict anastomotic leakage occurrence and, so far, there is no adequate preoperative bowel preparation that significantly helps to reduce its incidence [93]. Different preventive strategies have been proposed aimed at influencing factors that have been identified as impairing anastomotic healing [13-15]. Kirchhoff [16] for example, classified these factors into two categories: factors that can be influenced (e.g., obesity, nutritional status, preoperative bowel cleaning, experience of the surgeon) and predisposing or risk factors that are not under our control (e.g., age and gender). Strikingly, many of these risk factors have also been shown to affect the composition of the microbiota [17], while at the same time they can themselves be deeply impacted by the microbiota, suggesting that the gut microbiota has a role in intestinal wound healing [8].

In this study, we investigated in a *in vivo* mouse model whether administration of a next-generation beneficial bacteria prior to surgery could modulate the intestinal environment so that healing is accelerated, reducing the risk for anastomotic leakage, and enhancing post-surgery recovery in

general. We have recently developed a novel, fast-track, innovative intestinal wound healing model in mice called the 'colonoscopic leakage model', in which we create a controlled transmural perforation in the colon by using a biopsy grasper during a colonoscopy. This model was validated and showed its important advantages over the classical 'anastomotic leakage model', by decreasing the invasiveness [229]. *A. muciniphila* is a commensal bacterium that was isolated from the human gut microbiota in 2004 [163]. This bacterium represents between 0.5 and 5% of the microbial community in healthy subjects and is specialized in colonizing the mucus layer, being one of the few bacteria living close to the intestinal epithelial cells. This anaerobic, Gram-negative bacterium of the phylum Verrucomicrobia has been shown to digest mucus using mucolytic enzymes encoded in its genome [246]. *A. muciniphila* has been previously associated with beneficial metabolic effects on energy, glucose metabolism and gut barrier function [166] and is considered a next-generation beneficial bacterium. In a mechanistic point of view, we and others have previously discovered that *A. muciniphila* activates different receptors such as Toll-like receptor 2 (TLR2) and TLR4 [210, 247, 248] and modulates the immune system, thereby reinforcing the gut barrier [225 203 66]. It has also been shown that IL22 was upregulated by a treatment with *A. muciniphila* [249]. A disruption of the intestinal barrier upon injury to intestinal epithelial cells is known to lead to the exposure of numerous TLR ligands produced by commensals and eventually may induce intestinal inflammation but also peritonitis, and mice lacking TLR2, TLR4, or the myeloid differentiation primary response 88 (MyD88) are more sensitive to these diseases [83 229 250 251]. MyD88 is a central adaptor molecule for most TLRs, but more importantly MyD88 is involved in the control of IL22 expression and regulation. Given that *A. muciniphila* has been shown to activate different receptors and to modulate the Immune system and reinforce the gut barrier [247 251 252], and that IL22 was strongly upregulated by the treatment with *A.*

muciniphila, we investigated whether the beneficial effects of *A. muciniphila* on the wound healing were mechanistically dependent on the expression of MyD88 on the intestinal epithelial cells.

Here, we investigate its putative impact of *A. muciniphila* on host recovery and wound healing in the context of colorectal surgery in wild-type mice and also using mice with an inducible intestinal-epithelial-cell-specific deletion of MyD88. We also explored the direct impact of 2 h exposure to *A. muciniphila* on the expression of genes in the colon of human volunteers.

Materials and Methods

Ethics

All mouse experiments were approved by the Ethical Committee for Animal Care of the Health Sector of the Université Catholique de Louvain (UCLouvain), under number 2018/UCL/MD/013 and were performed in accordance with the guidelines of the Local Ethics Committee and in accordance with the Belgian Law of May 29, 2013, regarding the protection of laboratory animals (agreement number LA1230314).

The human study was approved by the Ethics Board of Uppsala/Örebro University. Colon biopsies were obtained from healthy subjects at the University Hospital Örebro, according to the study protocol from Brummer et al. The University Hospital Örebro Ethical committee (Dnr 2010/261) approved this study. Studies were conducted in full accordance with the principles of the 'Declaration of Helsinki' (52nd WMA General Assembly, Edinburgh, Scotland, October 2000). All subjects gave their written informed consent prior to their inclusion into the study.

Animals

Experiments 1 and 2 were designed to determine the effects of pretreating mice with A. muciniphila MucT (ATTC BAA-835) on body weight, organ weight, mortality, peritonitis severity, wound healing score, and different intestinal markers in the colonoscopic leakage model. For experiment 1 and 2 (replicate experiments performed independently), 9-weeks old male C57BL/6J mice were purchased from Janvier (Le Genest-Saint-Isle, France). The mice were housed in pairs under strict specific and opportunistic pathogen free (SOPF) conditions and in controlled environment (room

temperature of 22 ± 2 °C, 12-hour daylight cycle, lights off at 6 pm) with free access to food and water. After an acclimatization period of one week, mice were randomly assigned to one of two conditions: the control group, gavaged with vehicle (anaerobic PBS with 25% (vol/vol) glycerol) (CT, n=20 per experiment) or the test group gavaged with 200µl of a solution containing 1×10^9 live cells of *A. muciniphila* per ml (Akk, n=20 per experiment). Gavages were performed daily for three weeks in total. After two weeks of gavages, a colonoscopic controlled perforation was performed following the procedure described below (day 0). Gavages were continued until day 7 when mice were killed under anesthesia and tissues were collected (see below).

Experiment 3 aimed at investigating whether mice with an intestinal epithelial (IEC)-specific deletion of Myd88 (IEC-Myd88 knock-out (KO)) were developing a different phenotype than control mice on body weight, mortality, and peritonitis severity after transmural perforation using the colonoscopic leakage model. IEC-Myd88 KO mice were generated by crossing mice bearing a tamoxifen-dependent Cre recombinase expressed under the control of the villin promoter (Villin Cre-ERT2) with mice harboring a loxP-flanked Myd88 allele (C57BL/6 background, Jackson-Laboratory), as previously described [252]. The deletion was induced at 9 weeks of age by intra peritoneal (i.p.) injection of 100 µl tamoxifen (10 mg ml^{-1}) for 5 consecutive days. Tamoxifen was prepared by addition of ethanol to 50 mg of tamoxifen (tamoxifen-free base, MP Biomedicals) to obtain a 10 mg per 100 µl of tamoxifen suspension. A 10 mg ml^{-1} tamoxifen solution was prepared by addition of filtered sunflower oil, followed by 30 min sonication. The 10 mg ml^{-1} of tamoxifen solution was stored at 4 °C for up to 1 week.

The tamoxifen solution was sonicated just before use. In parallel, the Control group was injected intraperitoneally (i.p.) with 100 µl filtered sunflower oil for

5 consecutive days. Due to COVID-related restrictions, the colonic leakage procedure on these mice, as described below, was performed at the age of 22-weeks.

Experiment 4 aimed at investigating whether mice treated with *A. muciniphila* but lacking Myd88 specifically in the intestinal epithelial cells (IEC-Myd88 KO) were responding or not to the treatment and whether they developed a different phenotype than control mice on body weight, mortality, peritonitis severity, and intestinal markers in the colonoscopic leakage model. For this experiment, we used 11-week-old, male, IEC-Myd88 KO mice that we obtained as mentioned above, but with the usual wash out period. They were randomly assigned to one of two conditions: the control IEC-MyD88 KO group, gavaged with vehicle (IEC-Myd88 KO, n=20), or the test group gavaged with 200 µL of a solution containing 1×10^9 live cells of A. muciniphila per mL (IEC-Myd88 KO *Akkermansia*, n=17), with gavages performed for 2 weeks before proceeding to the colonoscopic controlled perforation (day 0). The mice were followed up closely and the gavages were continued for one week until the sacrifice (day 7), when the mice were killed under anesthesia and tissues were collected for analysis.

Colonoscopic Leakage Model

The colonoscopy leakage model was performed as described in Bachmann et al, Gut, 2020 [229]. This model has several advantages over the traditionally used “anastomotic leakage model” and was shown to decrease invasiveness and ameliorate recovery of the animals while remaining close to the human situation [229].

In brief, mice were anaesthetized using isoflurane gas (Forene, Abbott, Queenborough, Kent, England), in accordance with welfare criteria (3%

induction and of 2.5 % maintenance dose). A colonoscopy was performed, during which a transmural wounding was induced just before the left colic flexure (i.e., 3.5 cm ab anno and lateral in regard to the colonic mesentery with a diameter of 5 mm, analogues to the grasper jaws, which correspond to the gap between two stiches of a “bad” anastomosis) by using 3Fr-biopsy forceps. Mice quickly returned to their habitual environment and were observed while recovering. Mice that showed signs of severe discomfort or pain in the first few hours after the procedure were eliminated from analysis as this was considered to be a direct cause of the procedure, rather than of the transmural wound and they showed no overt sign of peritonitis (n=1 in the ^{1st} experiment, n=0 in the 2^{nd} experiment, n=0 in the 3^{rd} experiment, n=2 in the 4^{th}experiment). One of the advantages of using this technique, is that the mice do not need a preoperative bowel preparation, nor do they need to be fasted before the intervention.

Follow-up

Body weight was monitored before and every day after the procedure and pain/discomfort was assessed daily. Mice were sacrificed after 7 days, which corresponds to the last phase of wound-remodeling, during which the new epithelium is developed, and the final scar tissue is formed. In all our procedures, we abstained from using antibiotics either pre- or post-intervention.

Sacrifice/ Peritonitis Score

At sacrifice, 7 days after colonic perforation, we could distinguish different levels of severity of peritonitis and attribute a corresponding score:

0) mice in which no peritonitis was observed.

1) mice with a peritonitis that was localized only at the site of perforation and that did not result in death.

2) mice with a generalized peritonitis affecting the whole abdominal cavity, but that did not result in death.

3) mice that died before the end of the 7-day period that had only a localized peritonitis.

4) mice that died and displayed clear signs of a generalized peritonitis.

This scoring allowed for a sensitive appreciation of the outcome and a fast comparative evaluation of our experimental groups.

Tissue sampling

At the end of the experimental period, mice were anesthetized with isoflurane (Forene; Abbott) before exsanguination and tissue sampling, then mice were sacrificed by cervical dislocation. Liver, spleen, and caecum were precisely dissected, weighed.

After dissection of the colon and establishment of the peritonitis score, the zone of trauma was localized using a 20X binocular and samples were taken of three different locations: of the proximal colon (healthy colon), of the zone next to the perforation zone at a distance of 15 mm, and of the perforation zone itself.

In experiment 1, the colonic tissue collected of the three different zones was used for histological analysis, whereas in the second experiment it was used for qPCR analysis.

Histological analysis

For experiment 1, the thickness of the mucus layer was measured at the site of perforation and at a more distal, unaltered, section of the colon. The tissues were fixed in Carnoy's solution (ethanol 6: acid acetic 3: chloroform 1, v/v) for 24 h at room temperature. Samples were then immersed in ethanol 100 % for 24 h before processing for paraffin embedding and preparation of 5-µm tissue sections. Paraffin sections of 5µm were stained with alcian blue

[253]. Analysis was performed on at least 2 high-resolution whole-slide images per mouse and a minimum of 20 different measurements were made perpendicular to the inner mucus layer per field. Analyses were performed using ImageJ (version 1.48r, National Institutes of Health, Bethesda, Maryland, USA) in a blinded manner by two different observers.

For histopathological examination, sequenced sections every 100μm spanning the entire perforation site were stained with H&E and analyzed by an experienced pathologist in a blinded manner. For this purpose, we used the recently developed histological score proposed by Miltschitzky et al. [254]. This score takes into consideration parameters like "*Fibroblasts*", "*Inflammatory cells*", "*Blood vessel ingrowth*", "*Collagen formation*", in order to assess in which of the different phases of wound repair the tissue of the collected sample is located. This score permits to globally assess the evolution of the wound healing, by quantifying the "*Number of healed layers*", "*Epithelium closed*", "*Crypt architecture restored*" and determining the "*Overall healing quality*".

RNA preparation and real-time qPCR analysis

Total RNA was prepared from collected tissues using TriPure reagent (Roche, Basel, Switzerland). Quantification and integrity analysis of total RNA was performed by running 1 µL of each sample on an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit, Agilent, Santa Clara, CA, USA). cDNA was prepared by reverse transcription of 1 µg total RNA using a Reverse Transcription System Kit (Promega, Madison, WI, USA). Real-time PCR was performed with the CFX96 Real-time PCR system and CFX manager 3.1 software (BioRad, Hercules, CA, USA) using GoTaq qPCR Master Mix (Promega) for detection, according to the manufacturer's instructions. RPL19 RNA was chosen as the housekeeping gene, and data

were analyzed according to the $2^{-\Delta\Delta CT}$ method. The identity and purity of the amplified product were assessed by melting curve analysis at the end of amplification.

Proof-of-concept, study of local instillation of *A. muciniphila* in human healthy volunteers

In parallel with the rodent study, we performed an exploratory study of local instillation of 10^{11} cells/ day (in 100ml PBS) of the alive form of *Akkermansia* through a catheter placed with the aid of colonoscopy in the left colon, with continuously dripping of the liquid for two hours. The volunteers didn't have a colonic prep prior to the intervention. They were splited in two groups, one placebo (n=3) receiving only 100 ml PBS, and the other one (n=7) receiving A. muciniphila as previously described [223].

The design of the procedure was to start with a fecal sampling and a distal colonoscopy including mucosal biopsies, before placing the catheter in the distal descending colon region to administrate the live Akkermansia. After two hours of continuous administration, the catheter was removed, and new mucosal biopsy specimen were sampled in the same region. A last mucosal biopsy with stool sample collection was performed at one week after the perfusion procedure.

The biopsies were analyzed for total human transcriptome on microarrays, and they were used equally to quantify the *A. muciniphila* levels at the mucus level. The fecal samples were equally analyzed for *A. muciniphila* levels using qPCR and for total microbiota composition by 16S rRNA phylogenetic microarray. Detailed methods for the labelling and subsequent hybridizations of the arrays are described in the eukaryotic section of the GeneChip Expression Analysis Technical Manual, from Affymetrix (Affimetrix, Santa Clara, CA). The data can be found in GEO repository under the number

GSE211506
https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE211506).

Statistical analysis

The data are presented as the means ± s.e.m. The statistical significance of the difference was evaluated by one-way or two-way ANOVA followed by Tukey's post hoc multiple comparison test. The data with a superscript symbol, are significantly different. All the analyses were performed using GraphPad Prism version 9.00 for Mac (GraphPad Software, San Diego, CA). The presence of outliers was assessed using the Grubbs test.

Results

A. muciniphila treatment reduces mortality and peritonitis

The procedure (Figure 1A) resulted in only 1/80 peri-interventional mortality on a total of 80 mice (replicate experiments 1 and 2). The mice recovered fast and displayed normal behavior as soon as the anesthetics wore off. Body weight loss was limited, transient and did not differ between groups (Figure 1B). There were no overt differences in weights of liver, spleen nor caecum (Figure 1C).

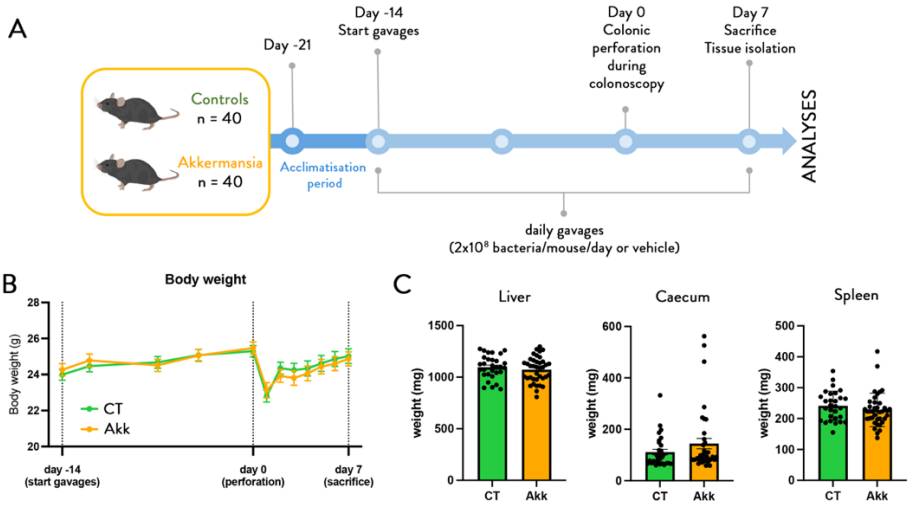


Figure 1. *A. muciniphila* does not affect body weight and organ weights. *(A) Experimental design (B) Body weight evolution before and after colonic perforation in control mice and mice treated with A. muciniphila. (C) weight of liver, caecum and spleen (n=40 per group) corresponding to the results of two independent experiments. Data are represented as mean +/- SEM.*

Mice treated with *A. muciniphila* had lower mortality rates (p=0.0224) (Figure 2A). At sacrifice, 7 days after colonic perforation, we could distinguish different levels of severity of peritonitis and attributed a corresponding score to each mouse. The reproducibility of this scoring was confirmed independently by two different examiners, blinded for the groups, without any difference in the evaluation. This showed a significant (P<0.0001) lower incidence of peritonitis for the *A. muciniphila* treated mice (Figure 2B). In the control group, 25% of the mice did not show any signs of peritonitis at day 7 (10/40 mice), whereas daily *A. muciniphila* administration significantly increased this number to 60% (24/40 mice). Among the mice that developed a peritonitis (75% for CT vs 40% for mice treated with *A. muciniphila*), 26%

was fatal in CT mice, whereas this number dropped to only 5% when mice were treated with *A. muciniphila*.

A. muciniphila improves wound healing without affecting the mucus layer thickness

Histological scoring of the wound healing process at the site of perforation after one week, showed an amelioration of the overall healing quality after *A. muciniphila* supplementation with a "*normal*" or "*good*" overall healing quality of 55 % in the *A. muciniphila*-treated mice as compared to only 30 % in the control group (P<0.0001) (Figure 2C). The percentage of wounds with a closed epithelium were 60 % (12/20 mice) in the *A. muciniphila*-treated mice and 40 % (8/20 mice) in control mice.

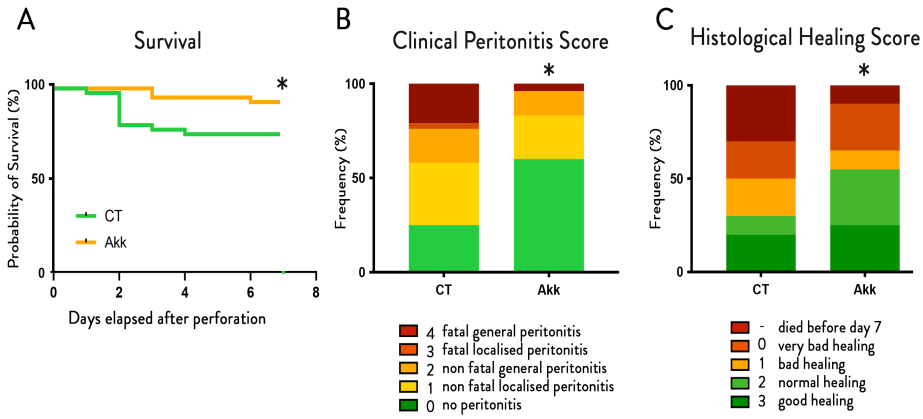


Figure 2. *A. muciniphila* administration reduces mortality, peritonitis severity and improves healing score.

Effect of A. muciniphila administration on (A) survival (n=40 per group) and (B) peritonitis se-verity (n=40 per group) and (C) healing score (n=20 per

*group) in a colonoscopic leakage model. Survival p-value = 0.0224 Gehan–Breslow–Wilcoxon test, clinical peritonitis score * corresponds to the statistics : p-value < 0.0001, and histological healing score p-value < 0.0001 Chi-square test.*

We analyzed the thickness of the mucus layer in both groups at two different sites: within the site of perforation and in a more distal, undisturbed part of the colon. This revealed no differences between the sites, nor groups (Figure 3), nor did we find any differences in expression profiles of Muc2, a marker for mucus production. Quantification of this mucus layer thickness revealed no differences between the sites, nor groups (Figure 3B), nor did we find any differences in expression profiles of Muc2. (Figure 3C).

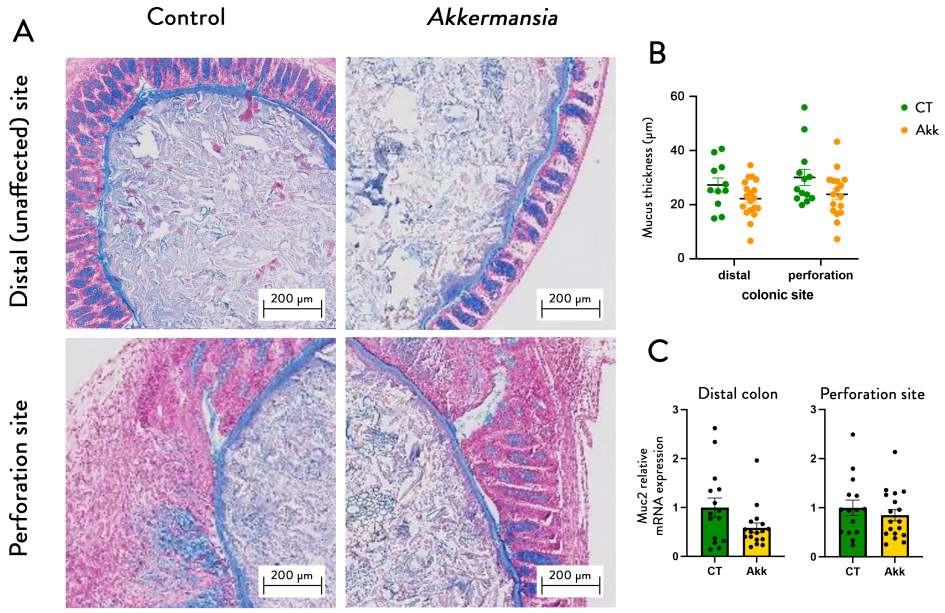


Figure 3. *A. muciniphila* administration does not affect mucus thickness and *Muc2* expression

(A) *Representative Alcian blue-stained images of the mucus layer in the distal colon and the per-foration site of mice treated with or without A. muciniphila.* ***(B)*** *Mucus thickness measurements****.*** ***(C)*** *Relative mRNA expression of Muc2. Data are represented as mean +/− SEM and black dots (dotplots) represent individual values for each animal. Statistical difference assessed using non-parametric Mann–Whitney test, p-value > 0.05.*

A. muciniphila increases the expression of IL22 in the colon

IL22 is a cytokine immediately produced to initiate immune response against gut barrier impairment and is known as a key mediator involved in wound healing, tissue regeneration and mucous production thereby acting as a protective factor against pathogens [255]. Moreover, *A. muciniphila* has been shown to modulate the immune system [226] by acting on the toll-like receptor 2 (TLR2) [247]. Interestingly, we found that *A. muciniphila* significantly upregulated the expression of IL22 by 400% and doubled the expression of IL17A (Figure 4A) although this effect did not reached significance. The other markers were not affected by the treatment (Figure 4A).

Next, we analyzed different relevant markers of gut barrier integrity including antimicrobial peptides (Reg3γ, Lyz1, Ang1, Pla2g2) and markers of intestinal cell renewal, differentiation, and proliferation (CCND1, Intectin, Math1, NGN3 and NeuroD1) but found no differences in gene expression between both groups, both at the unharmed distal part of the colon (not shown) and at the site of perforation (Figure 4B-C). We also found no effect on Collagen 1A (COL1A) and matrix metalloproteinase 2 (MMP2), which is involved in extracellular remodeling (Figure 4D).

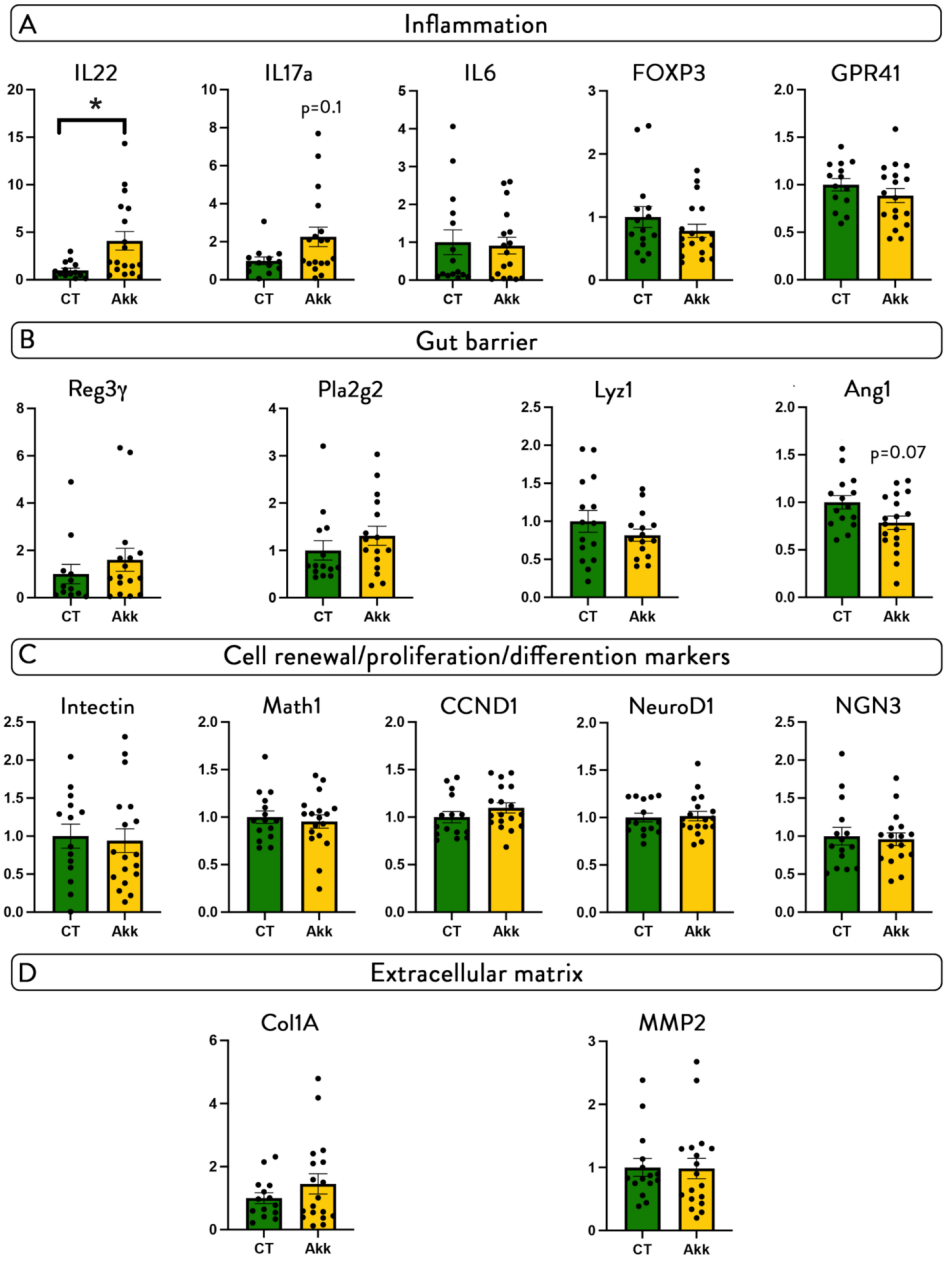


Figure 4. A. muciniphila administration increases IL22 and IL17 without affecting other markers of inflammation, gut barrier, cell renewal,

proliferation markers, and matrix formation. *Relative mRNA expression of markers of* ***(A)*** *inflammation,* ***(B)*** *gut barrier integrity,* **(C)** *cell renewal, proliferation, and differentiation, (D) extracellular matrix at the site of perforation. Data are shown as mean ± SEM, black dots (dotplots) represent individual values for each animal; * p-values were obtained after non-parametric Mann–Whitney test.*

A. muciniphila reduces mortality, peritonitis and improves wound healing by an intestinal epithelial cells specific MyD88-dependent mechanism

The intestinal innate immune system is one of the most important factors involved in the interactions between gut microbes and host. Recognition of pathogen-associated molecular patterns by the intestinal epithelial cells is important. MyD88 (myeloid differentiation primary response gene 88) is a central adaptor molecule for most Toll-like receptors (TLRs) and is also involved in the control of IL22 expression and regulation. Given that *A. muciniphila* has been shown to activate different receptors of the innate immune system including TLR2 [203, 225] and that IL22 was strongly upregulated by the treatment with *A. muciniphila,* we investigated whether the beneficial effects of *A. muciniphila* on the wound healing were mechanistically dependent on the expression of Myd88 on the intestinal epithelial cells.

To this aim, we used mice with an inducible intestinal epithelial cell-specific deletion of MyD88. In a first experiment (Figure 5A), we determined whether the intestinal epithelial cell-specific inactivation of Myd88 itself had any effect on body weight, survival, and peritonitis severity. We found that intestinal epithelial cell-specific deletion of MyD88 did not affect the development of peritonitis and no differences between both genotypes were observed for any of the parameters tested (Figure 5B-D).

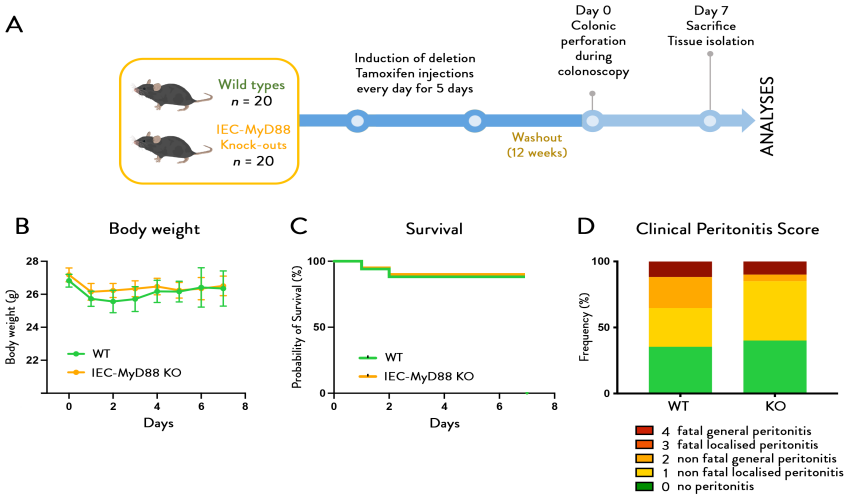


Figure 5. Mice with intestinal epithelial cell-specific deletion of Myd88 are similarly sensitive as wild-type mice to mortality and peritonitis.
(A) *Experimental design.* ***(B)*** *Body weight evolution,* ***(C)*** *survival rates and* ***(D)*** *peritonitis scores after colonic perforation in control mice and mice with an intestinal epithelial cell-specific deletion of Myd88 (IEC-villinMYD88 Knock-outs (n=20 per group). Data are shown as mean ± SEM. Probability of survival assessed using Gehan–Breslow–Wilcoxon test and Chi-square test for clinical peritonitis score, p-value > 0.05.*

Next, we determined whether *A. muciniphila* could still ameliorate the phenotype of IEC-Myd88 KO mice (Figure 6A-B). We found that daily administration of *A. muciniphila* did not improve survival (Figure 6C) or peritonitis scores and the Akkermansia treated mice had a worse incidence of developing peritonitis then the non-treated ones (Figure 6D) in mice lacking intestinal MYD88, thereby showing that *A. muciniphila* needs a functional intestinal epithial Myd88 protein to exert its protective effects. In

addition, we found *A. muciniphila* treatment failed to increase both IL22 and IL17 (Figure 6E), thereby also showing that mice lacking intestinal MYD88 are not responding to the beneficial effects of *A. muciniphila.*

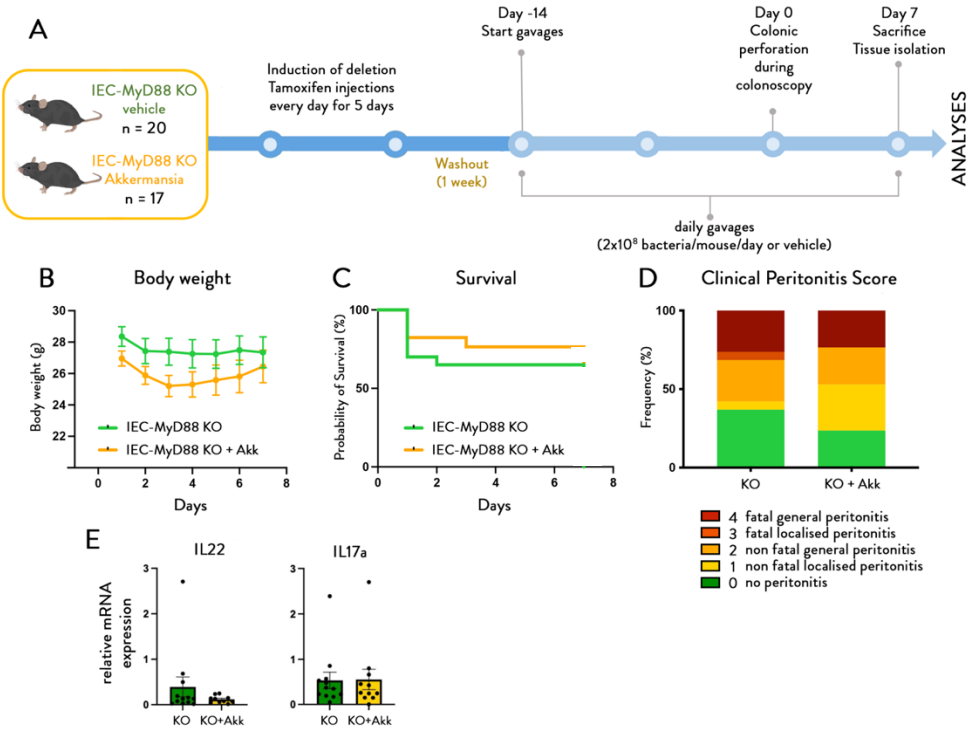


Figure 6. Mice with intestinal epithelial cell-specific deletion of Myd88 are not responding to the beneficial effects of *A. muciniphila* on mortality and peritonitis.

(A) Experimental design. (B) Body weight evolution, (C) survival rates, and (D) peritonitis scores after colonic perforation. (E) IL22 and IL17 in the colon of mice with an intestinal-epithelial-cell-specific deletion of Myd88 (IEC-MYD88 KO) with or without treatment with A. muciniphila (n=20 per group). Data are shown as mean ± SEM, black dots (dotplots) represent individual values for each animal. Probability of survival assessed using Gehan–Breslow–Wilcoxon test and Chi-square test for clinical peritonitis score, p-

value > 0.05. Non-parametric Mann–Whitney test used to assess IL22 and IL17, p-value > 0.05.

A. muciniphila Changes the Expression of Genes Involved in Cell Cycling, Gene Transcription, and Immune and Inflammatory Pathways in the Colon of Healthy Humans

Although the data obtained in rodents are interesting, the present findings should be confirmed in humans. However, before starting any large-scale clinical trial, we need to verify whether A. muciniphila can directly affect the expression of genes in the colonic mucosa and whether the exposure to A. muciniphila is well tolerated and does not induce any adverse events. Therefore, we performed a proof-of-concept, exploratory, study in healthy humans. Our data showed that the local intestinal instillation of the A. muciniphila is not associated with any negative effects on the subjects, none of them experienced any side effects.

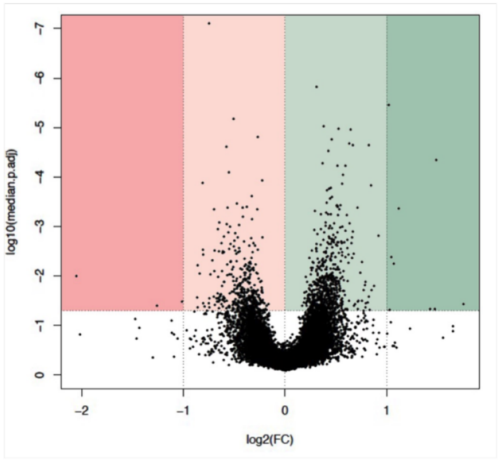
The quantitative analysis of the *A. muciniphila* levels did not show an effect in the fecal samples but, interestingly, an increased level at the mucus layer, in the mucosal biopsies taken at two hours after start of perfusion, but not persisting in the samples taken after one week (not shown). There was no significant effect found when analyzing the fecal microbiota composition prior and after *A. muciniphila* instillation. Interestingly, by using a transcriptomic analysis, we found that numerous genes were decreased or increased due to the *A. muciniphila* treatment, mostly genes involved in cell cycling, gene transcription, and immune and inflammatory pathways, but also in metabolic pathways of the intestinal cells (Figure 7 and Supplementary Table S1). More specifically, among the specific genes increased *by A. muciniphila*, we found a significant increase in genes related to the regulation of immunity and inflammation. For instance, TRIM40 (tripartite motif containing 40) is a gene

described to be involved in the regulation of the activity of the TLR4 signaling pathway and NF-κB (nuclear factor-kappa B) [256] (Figure 7). TRIM40 is known to promote the neddylation of the inhibitor of NF-κB subunit gamma, a crucial regulator for NF-κB activation, and as a consequence leads to strong inhibition of the NF-κB activity [257]. Along the same line, we found that the expression of the gene coding for MARCO (macrophage receptor with collagenous structure) was significantly increased (Supplementary Table S1). This marker is a scavenger receptor expressed on the cell surface of macrophages that mediates opsonin-independent phagocytosis. It has also been sug-gested that MARCO regulates the activation status of the immune system and can be involved in balancing a proper immune response to infection. This regulation could also be important in maintaining tolerance [258] [259]. We also identified numerous changes in the expression of the gene coding for gene transcription regulation, energy metabolism, and cell cycling (e.g., regulation of alternative splicing, ribosome biogenesis, tubulin formation, transporters). These data are in line with previous studies in rodents showing that the monocolonization of mice with *A. muciniphila* modulates the expression of different colonic genes [226].

A

T = 0h
T = 2h
T = 1 week
Perfusion
(2 hours)
10¹¹ cells in
100 ml PBS
Akkermansia
N = 7
Placebo (PBS)
N = 3
No Negative Effects
Biopsy
Biopsy
Biopsy

B


log10(median.p.adj)
log2(FC)

C

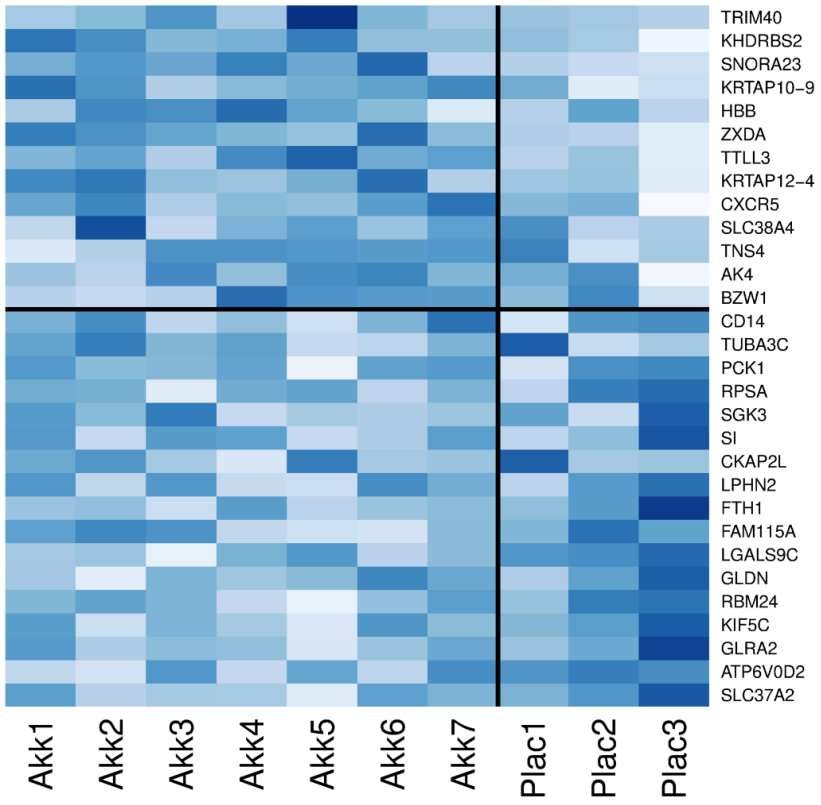

TRIM40
KHDRBS2
SNORA23
KRTAP10-9
HBB
ZXDA
TTLL3
KRTAP12-4
CXCR5
SLC38A4
TNS4
AK4
BZW1
CD14
TUBA3C
PCK1
RPSA
SGK3
SI
CKAP2L
LPHN2
FTH1
FAM115A
LGALS9C
GLDN
RBM24
KIF5C
GLRA2
ATP6V0D2
SLC37A2
Akk1
Akk2
Akk3
Akk4
Akk5
Akk6
Akk7
Plac1
Plac2
Plac3

Figure 7. Proof-of-concept study in human Healthy human subjects treated 2 hours with *A. muciniphila* on gene expression versus baseline treatment and placebo.

(A) *Experimental design and volcano plot showing transcriptional analysis on colonic biopsies after 2 h perfusion versus basal.* ***(B)*** *Dark and light pink left parts of the volcano plot represent the genes downregulated 2 h after exposure to A. muciniphila, whereas the dark and light green on the right part represent the genes upregulated 2 h after exposure to A. muciniphila. Both dark pink and dark green parts are showing genes for which the expression is changed by a magnitude of at least 2-fold after 2 h perfusion with A. muciniphila versus basal (i.e., before exposure).* ***(C)*** *The heatmap is based on the 30 genes displaying significant (adj-p values smaller than 0.05) and largest absolute fold changes in the comparison A. muciniphila (Akk) vs. placebo (Plac). In the heatmap, the genes presented above the horizontal line represent upregulation of A. muciniphila vs. placebo while the genes presented below the horizontal line represent downregulation of A. muciniphila vs. placebo. Every single column represents one subject, i.e., exposed to A. muciniphila (left of the vertical line) or exposed to placebo (right of the vertical line). The genes changed, their levels of expression, names, and statistical changes are shown in Supplementary Table S1.*

Discussion

More and more studies suggest that the local intestinal microbiota play an influential role during healing of a colonic anastomosis [153]. A recent metanalysis focusing on clinical trials, studying the impact of the pre- and postoperative administration of disponible pre- and probiotics showed positive results with a reduction of the length of stay in hospital as well as

lower rates of surgical site infections. Unfortunately, these measures were not sufficient to avoid colonic anastomotic leakages [260] and the need for adequate interventions remains. For this reason, we decided to test the impact of the administration of *Akkermansia muciniphila* using the newly developed "colonoscopic leakage model".

We found that the administration of *A. muciniphila* two weeks prior to and one week after the operative intervention resulted in a significant amelioration of the survival rate and was associated with lower rates of peritonitis and less severe peritonitis. Histological examination revealed considerable improvement of healing outcomes and these effects were linked to higher IL22 production. More importantly, we performed a first proof-of-concept study in human volunteers by instilling *A. muciniphila* directly in the colon and measuring transcriptomic markers in colonic biopsies 2hours after the continuous exposure to the bacterium. Our data suggest that *A. muciniphila* is safe, can rapidly induce changes in the expression of genes in the colonic mucosa exposed to *A. muciniphila*.

There have been several mechanisms described by which *A. muciniphila* can exert its beneficial effects and affect host physiology. These include improvement of the gut barrier function [210, 225], immunomodulation of the adaptive immune system [226], and activation of anti-inflammatory responses [227]. *A. muciniphila* has previously been proposed to restore mucin production [228] by being involved in a positive feedback loop, whereby through mucin degradation it also stimulates mucin production. Therefore, we measured mucus thickness in our mice to evaluate whether this might contribute to an improved gut barrier function and have a protective effect on wound healing. However, we found no impact of *A. muciniphila* administration on mucus production. Nevertheless, it should be noted that the studies showing a restoration of the mucus production after the administration of *A. muciniphila* have been done in mice fed high-fat diet [66,

[167] and aging mice [76]. In this context, the mucus production is impaired, and the mucus layer is thinner. In the present study, the mucus layer thickness is comparable to that in a normal colon [70].

In addition to the physical protection conferred by the mucus layer to keep pathogens from getting into the body and helping with the wound healing, the biochemical barrier is another important mechanism that helps keep commensal bacteria at a distance and fend off pathogenic bacteria. Specific antimicrobial peptides (AMPs) [207] such as regenerating islet derived-3 gamma (Reg3γ), phospholipase A2 group II (Pla2g2), Lysozyme C (Lyz1) and angiogenin 1 (Angg1) act as a firewall against certain bacteria by affecting their viability and are involved in intestinal epithelial cell turnover [186]. However, our analysis of several relevant markers of gut barrier integrity did not show any differences between treated and untreated mice.

Although we found no effects on markers of gut barrier integrity, cell renewal or extracellular matrix, we found an increase of IL22 and IL17a expression at the site of perforation. IL22 has been shown to contribute to epithelial cell expansion, proliferation, AMP production and to improve barrier dysfunction in human and mouse models [255].

Like *A. muciniphila* effect, mucins are highly inducible by IL22 and constitute the thick mucosal layer that coats the epithelium and that is impenetrable to many commensal bacteria, thereby limiting their potential to cause inflammation [261]. IL22 has been shown to upregulate several mucus associated genes, such as MUC1, MUC3, MUC10, and MUC13 in colonic epithelial cells [262].

In acute phase, IL22 is known to recruit neutrophils toward the site of infection as an inflammatory response [263]. It may act independently but it can also synergize with other cytokines like IL17 to elicit its role [264]. Regenerating islet-derived protein 3γ (Reg3γ) mRNA expression in enterocytes and Paneth cells is controlled by microorganism-associated molecular patterns

through Toll-like receptors (TLRs) and is dependent on the TLR signaling adaptor myeloid differentiation primary response protein 88 (MYD88) [265]. *Akkermansia muciniphila* had previously been positively correlated with intestinal expression of Reg3γ [66, 186], a finding that we could not confirm in this study. However, the previous observations were made in the context of a high-fat diet and a downregulation of this marker. *A. muciniphila* has been proposed as a mediator of Myd88/TLR2 innate immune signaling and some evidence indicates that Myd88 is involved in the modulation of wound healing [266, 267], but the underlying mechanism is still unclear. It was previously demonstrated that the TLR-induced secretion of IL22 requires Myd88 signaling and that a strong reduction of IL22 in Myd88 -/- was observed [268]. In line with this observation, our experiments using inducible intestinal epithelial-cell specific deletion of MyD88, revealed that *A. muciniphila* loses its beneficial effects if MYD88 is absent, suggesting that this adapter protein is required for transfer of *A. muciniphila* signaling.

Although *A. muciniphila* seems to play an important role in the in the better outcome observed in our model, we cannot exclude that the local microbiota may also be involved by influencing the production of the intestinal antimicrobial proteins (AMPs) independently from this pathway [269], for example through the production of the short chain fatty acid butyrate in the colon through microbial fermentation of dietary fibre [270].

Finally, by using a proof-of-concept approach we administer *A. muciniphila* directly in the colon of human volunteers for two hours. By using mucosal biopsies before, two hours and 1 week after the initial exposure to *A. muciniphila* versus placebo, we found that the administration of the bacteria did not elicit any specific adverse events and modulates the expression of several genes involved in the regulation of immunity, gut barrier and host metabolism. However, it is worth noting that among the genes induced by the exposition to *A. muciniphila*, several genes were

identified that are under direct control of the TLR/MyD88/NF-κB pathways (e.g., higher TRIM40 and MARCO).

In addition, the safety profile observed here is in line with another human study showing that oral administration for 3 months of either live or pasteurized A. muciniphila is safe and well tolerated [203] [223]. Therefore, our findings could be relevant for future human interventions. Although this pilot study is underpowered, this first exploratory study will be useful to design future prospective interventional studies to investigate the putative wound healing effects of *A. muciniphila* on the human colon.

We have previously found in both mice and humans that *A. muciniphila* treatment was not associated with a specific change in the gut microbiota composition [66] [223]. Although the microbiota was not assessed in the mouse study described here, we spec-ulate that there are likely no specific changes in the microbiota, since we also found in the present human intervention that the exposure to *A. muciniphila* was indeed enriching the mucosa of the subject *in A. muciniphila* after the two hours of exposure, and this effect was transient and also did not affect the overall microbiota composition of the subjects (not shown). This is perfectly in line with our previous human study showing that the oral administration of *A. muciniphila* for 3 months of either live or pasteurized *A. muciniphila* did not affect the overall microbiota composition measured by sequencing [223].

Our animal model, which showed a significant amelioration of the intestinal wound healing, is based on a pre-operative administration of *A. muciniphila* for 2 weeks. The current standard prehabilitation program (which is meant to improve the preoperative status of the patients with the help of physiotherapy, nutrition improvement and psychological counseling) has a standard duration of 3 weeks and showed some improvement on the outcome, but unfortunately no amelioration of the AL rate [22]. If our results should prove to be translatable also to humans, it could be envisaged to add

A. muciniphila supplementation to the existing protocol of prehabilitation, without delaying the timeline from diagnosis to operation.

Conclusions

In conclusion, administrating live A. muciniphila improves wound healing of a transmural colonic wall defect (i.e colonic anastomosis) in a mouse model through a mechanism requiring the MyD88-dependent pathway and possibly requiring IL22 signaling (Figure 8). Regarding the proof-of-concept study performed on human volunteers, we can conclude that in healthy human subjects the local administration of A. muciniphila is well tolerated and significantly changes the expression of genes involved in the immune pathways. This study illustrates how understanding the complex interactions between host and a specific potential beneficial microbe may support developing future therapeutic strategies targeting the gut microbiota. However, the question remains if we can extrapolate these in vivo animal findings to humans and additional experiments in humans are therefore inevitable.

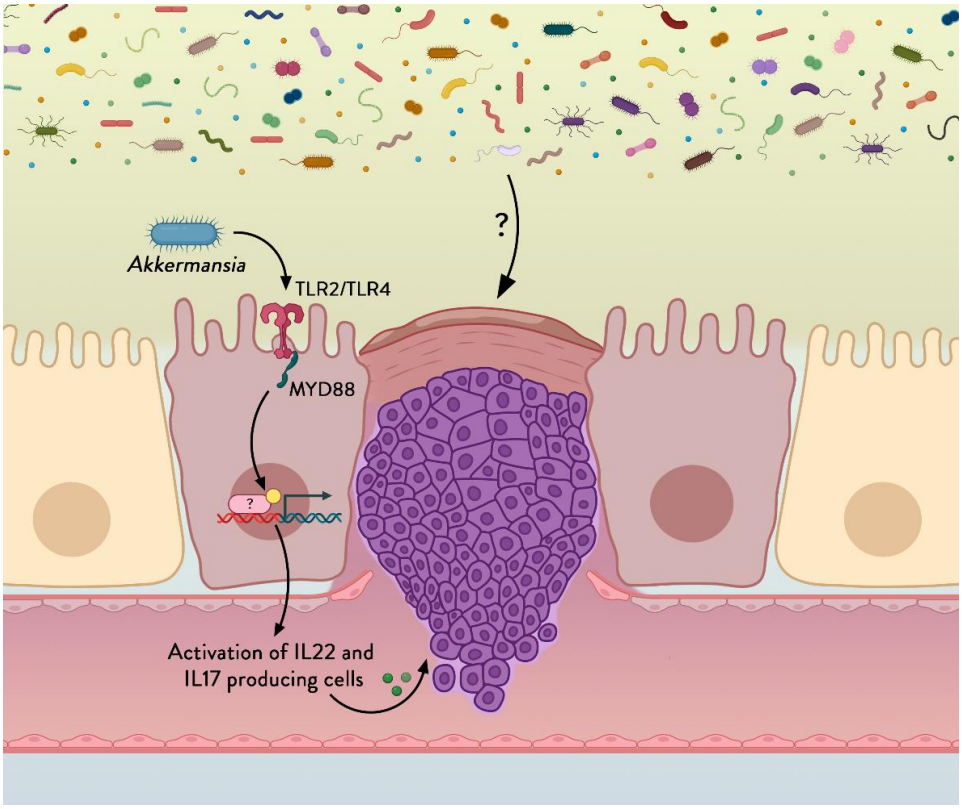


Figure 8: Graphical abstract. Working and mechanistic model proposed

A. muciniphila activates Toll-like receptors (TLRs) and a signal is transferred via the adaptor-protein MYD88 leading to the expression of certain yet unknown mediators that activate the production of IL22 and IL17, which in turn results in a, improved wound healing and better general outcome. (?) depicted potential other unidentified signals from the gut microbiota that may also influence this process.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Supplementary Table S1: Genes changed, their levels of expression, names and statistical changes, Supplementary Figure S1: Expression of TLR2 and TLR4 in the colon of mice.

Author Contributions: Conceptualization, P.D.C., R.B., and M.V.H.; methodology, P.D.C., W.M.d.V., M.V.H., R.B., P.D.C., R.J.B., I.R., D.P., C.B., and J.P.O.; validation, P.D.C. and W.M.d.V.; formal analysis investigation, M.V.H., R.B., P.B., P.D.C., W.M.d.V., R.J.B., I.R., C.B., and J.P.O.; re-sources, N.M.D., D.L., A.K., P.D.C., D.P., W.M.d.V., and R.J.B.; data curation, P.D.C., R.B., and M.V.H.; writing—original draft preparation, P.D.C., R.B., and M.V.H.; writing—review and ed-iting, all authors; supervision, P.D.C.; project administration, P.D.C. and W.M.d.V.; funding acqui-sition, P.D.C. and W.M.d.V. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The human study was approved by the Ethics Board of Uppsala/Örebro University. Colon biopsies were obtained from healthy subjects at the University Hospital Örebro, according to the study protocol from Brummer et al. The University Hospital Örebro Ethical committee protocol code Dnr 2010/261 approval 2010 approved this study. Stud-ies were conducted in full accordance with the principles of the Declaration of Helsinki (52nd WMA General Assembly, Edinburgh, Scotland, October 2000).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. All subjects gave their written informed consent prior to their inclusion into the study and to use their data and to publish papers.

Data Availability Statement: All the details regarding analytic methods and study are made available to other researchers as Supplementary Table S1.
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Conflicts of Interest: P.D.C. and W.M.d.V. are inventors on patent applications dealing with the use of A. muciniphila and its components in the treatment of metabolic disorders. P.D.C. and W.M.d.V. are co-founders of The Akkermansia Company. P.D.C. is the cofounder of Enterosys. W.M.d.V. is the co-founder of Caelus Health and inventor on patents on the use of Eubacterium hallii. The other authors have no conflict of interest to declare. The funders had no role in the de-sign of the study; in the collection, analyses, or interpretation of data; in the writing of the manu-script; or in the decision to publish the results.

GENERAL DISCUSSION AND PERSPECTIVES

In this thesis we delved deeper into the impact of the gut microbiota on wound healing. We developed the *colonoscopic leakage model* as a new tool that can be used as an alternative to the current models proposed in the literature to study the mechanisms of intestinal wound healing. These models are mainly dependent on a complete surgical procedure of anastomosis. The most interesting of these model is the one developed by Pommergaard [230]. It is performed by adapting the number of intestinal anastomotic sutures after segmental colonic resection: while 8 stitches do normally not give rise to anastomotic leakages (control group), reducing this to 5 stiches achieves a probability of developing leakage and peritonitis in 50% of the anastomotic leakage group.

While we were able to successfully reproduce this model in our laboratory and achieved the same outcome, we discovered that although being feasible and reproductible, this model had numerous drawbacks that made it unsuited for the purpose of our research and limits the use of this model for potential translational approaches. More specifically, Pommergaard's model displayed an important occurrence of occlusions in both the control and anastomotic leakage group, thereby profoundly affecting the intestinal transit and making it problematic to perform local interventions and changes of the microbiota in the colon. These complications, which are specific to small animals and do not occur in humans, make it necessary to exclude almost half of the animals in the study. In addition, this model is extremely time consuming, requiring about 45-60 min per animal, and is very invasive, causing animals to lose significant weight and recover slowly, often never returning to their initial body weights within the time window of 7 days. After numerous failures in trying to optimize the conditions and improve the model, we decided to abandon it altogether and to develop our own. This led us to "*the colonic leakage model*". In this procedure, we create a controlled

transmural perforation in a normal vascularized colon by using a grasper that is introduced into the colon via an endoscope. The entire procedure takes less than 10 minutes, about 6 times faster than the anastomosis model. Importantly, the mice displayed a normal behaviour as soon as the anaesthetics wore off and required low use of pain killers without the necessity of using opiates. The overall weight loss was very limited, and mice returned to their preoperative body weights in less than 5 days and we did not observe any signs of intestinal occlusion.

Because of these advantages over the classical anastomotic models, we believe our colonoscopic leakage model is goes beyond is better suited to study intestinal wound healing at the local level and even allows using colonoscopic imaging. Also, this model has a clear potential for clinical use and can offer researchers and clinicians an easy model to study intestinal wound healing and investigate the effect of specific treatments (i.e., drugs and nutritional approaches).

To further validate our newly developed wound healing model, we decided to test it under pathological conditions in which the healing process is known to be compromised. Obesity is such a condition and many human studies have shown that excessive overweight is associated with an incremental independent morbidity risk, especially for surgical site infections but also for anastomotic leakage [258, 259]. Therefore, we subjected mice with genetically induced obesity (*i.e* mice with a mutation in the gene responsible for leptin production, referred to as *ob/ob mice*) [260] to our colonic leakage protocol.
We found wound healing in *ob/ob mice* to be less efficient than in control mice, as evidenced by the higher mortality rates and the higher prevalence of peritonitis in these severely obese animals (Figure 1). While we did not go deeper into the underlying mechanisms at this point, it is possible that the

differences in gut microbiota compositions, which have been described often between these obese mice and their lean counterparts, play a role. It would therefore be interesting to test whether modulating this microbiota might improve colonic wound healing.

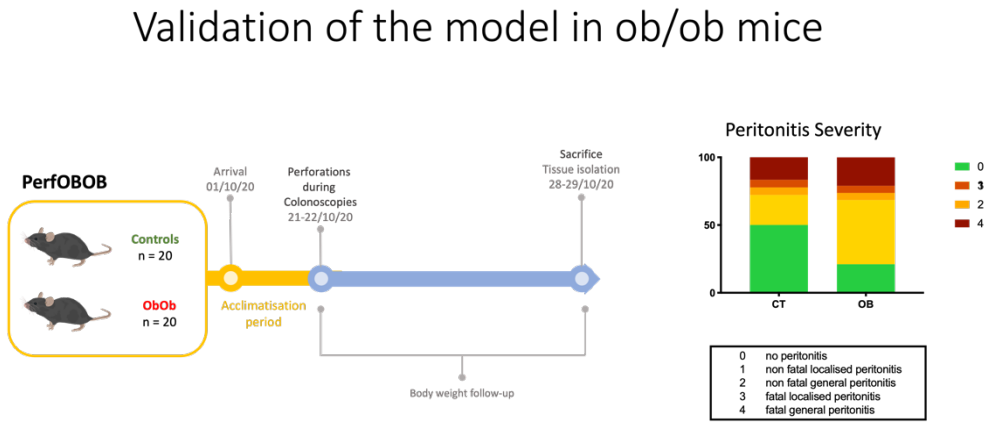

Figure 1: ***Obesity slows down the wound healing process*** *in the gut leading to more peritonitis*

In a clinical environment we aim to perform the best possible anastomosis to prevent the occurrence of an anastomotic leakage. Despite this, because of different factors, anastomotic leakage still happens. On the other hand, in experimental settings, the aim of using an anastomotic leakage model is to ensure a stable number of anastomotic leakages to be compared against a control group and this stable number is reached either by performing a technically insufficient anastomosis or by impairing the vascularisation of the anastomotic tissue. Even if the anastomotic leakage models do not simulate exactly the clinical conditions, they enable the systematic analysis of the different factors involved in clinical environment in the occurrence of anastomotic leakage. Similarly, even if different from the clinical anastomotic

leakage settings, the described colonoscopic leakage model allows to analyse the factors involved in the occurrence of an anastomotic leakage in a standardised manner, while presenting at the same time some advantages compared to the classical anastomotic leakage models.

In the second chapter of this thesis, we aimed at investigating whether *A. muciniphila* enhances gut barrier function, wound healing and reduces peritonitis and mortality in our colonoscopic leakage model. This kind of intervention would be of interest in the context of reducing complications following colorectal surgery. As detailed in the introduction and in the aims of this thesis, anastomotic leakage is a major complication that has been linked to high morbidity including peritonitis, increasing the risk of death. In addition, because it was not known whether *A. muciniphila* was able to modulate gene expression in the colonic mucosa of humans, we also designed a pilot study to investigate this question.
We found that *A. muciniphila* improved wound healing following colonic perforation, thereby improving peritonitis scores and reducing mortality. This was associated with changes in IL22 and IL17 production. To further explore whether *A. muciniphila* requires specific signaling pathways, we studied the role of TLR/MyD88 signaling in the healing process using IEC-MyD88 KO mouse models. Using this mouse model, we showed that *A. muciniphila* modulated its effects at least in part through a MyD88-mediated pathway. Finally, we also demonstrated that *A. muciniphila* instillation in the colon of healthy subject was sufficient to modulate the expression of several genes within a 2 hours period.

Although the effect of *A. muciniphila* is rather straightforward and clearly demonstrates a potential to improve health, it remains to be determined how this compares with other known beneficial bacteria, such as for instance

Bifidobacterium longum [271, 272]. Alam et al. have also discovered that *Lacticaseibacillus rhamnosus* GG (formerly named *Lactobacillus rhamnosus* GG) improved wound healing occurring at the surface of the epithelium, but this has never been tested in our complex and more invasive model [153, 273]. It remains to be determined whether *L. rhamnosus* GG could also reduce the mortality and peritonitis score in our model. However, it is clear that not all new generation beneficial bacteria are by default able to act on wound healing. This was demonstrated testing a butyrate producing bacteria (i.e., *Dysosmobacter welbionis*) [274], that, despite the fact that butyrate is known to potentially increase the intestinal epithelial cells renewal and has anti-inflammatory properties [275, 276], did not have any effect (data not presented in this thesis). While higher doses of *D. welbionis* might be needed to obtain a response, it is also possible that the observed effects on wound healing are specific to *A. muciniphila*.

Although we found a strong and reproducible effect of *A. muciniphila* on lowering mortality/peritonitis and improving wound healing and were able to link this to IL22 and IL17, we could not detect any specific changes on any other markers of inflammation, gut barrier, cell renewal, proliferation markers, mucus production and thickness and matrix formation. This can be due to the fact that we have analyzed the samples after 7 days of recovery and many of the initial effects might have resolved by then. Therefore, an important perspective would be to sacrifice mice at different time points follow-up experiments in order to fully cover the period of wound healing and characterize each of the specific phases (the haemostasis phase, the inflammatory phase, the proliferative phase and the wound remodeling phase). For this, we could plan a time course, starting from pre-perforation to the time of perforation and add at least three time points after perforation. This approach, while technically feasible, will require a large number of additional animals, if we do not want to miss out on the endpoints related to

mortality and peritonitis outcomes. Nevertheless, it will help answer some interesting questions as it is presumed significant inflammation takes place immediately (within 24-48 h) post perforation. For example, IL-6 has been shown to be induced immediately after perforation injury [277]. Furthermore, *A. muciniphila* supplementation has been shown to increase peritoneal resident macrophages and decrease Ly6Cint monocyte frequencies in spleen and mesenteric lymph nodes, coupled with activated CD80+CD273- B cells in Peyer's patches [76]. As detailed here above, the fact that we observed few significant gene expression changes but mostly an impact on IL22 expression could be also criticized or viewed as a bias due to the methodology used and therefore the targeted approach. Therefore, it would be interesting to use an unbiased approach (e.g., cytokine arrays, sequencing methods, proteomics) and test additional time points as discussed above. Indeed, we may not rule out that some genes required for gut barrier, cell renewal, proliferation, differentiation and extracellular matrix are expressed differently at different time points.

Another potential interesting approach would be to use single cell sequencing to determine changes in immune cell population both in the perforated and unaffected colon of *A. muciniphila* supplemented mice as well as in IEC-MyD88 KO mice and in their respective controls. Also, given that Alam et al[153] have shown that the local microbiota could be affected by the wound healing process, it could be of interest to better define the microbiota composition at the site of the wound healing and at a more distant unaffected site, both in mice treated with *A. muciniphila* and in KO mice (and their controls).

Although we have demonstrated that mice lacking MyD88 specifically in the intestinal epithelial cells are not responding differently to the development of peritonitis and mortality as in the WT mice, we may not exclude that these

mice have different gut microbiota composition at the level of the mucosa. Indeed, we previously discovered that IEC-MyD88 KO mice have a different response to high-fat diet induced changes in metabolism and gut microbiota[252]. Therefore, whether these tissue specific KO mice are also displaying a different response in the perforation-induced changes in mucosal microbiota remains to be proven. Having said that, we unequivocally showed that mice lacking Myd88 in the intestinal epithelium were not protected by the administration of *A. muciniphila* and strongly support the link between the activation of TLR/MyD88 pathways to improve the *A. muciniphila*-induced wound healing. Nevertheless, we acknowledge that the overall mechanistic aspects linking *A. muciniphila* – IL22 – wound healing are not yet sufficiently convincing and propose further experiments to address some remaining questions. For instance, can IL22 administration phenocopy *A. muciniphila* effects? Do mice lacking IL22 develop higher mortality or peritonitis than the WT mice? Do mice lacking IL22 respond to *A. muciniphila*? Only after these questions have been answered, will the role of IL22 become clear.

Both synthetic and biological TLR-agonists are already used in clinical routine in oncology due to their potent stimulation of innate and adaptive immunity as well as their effects on the tumor microenvironment [278]. For example, a live attenuated *Mycobacterium bovis* strain, known to stimulate TLR2 and TLR4 via its mycobacterial components, is approved and used for intravesical therapy of bladder carcinoma in situ and superficial bladder cancers [279]. One interesting idea would be to check, in animal settings, whether the administration of TLR2 / TLR 4 agonist replicates the beneficial effects of the administration of *A. muciniphila* on the intestinal wound healing. It is worth noting that all these approved TLR's agonist are used as topical medication, in direct contact with the carcinoma. It remains to be investigated whether the administration of *A. muciniphila* is also positively acting via both

systemic and local contact with the mucosa. This could be done for example by testing local administration versus oral gavage (prehabilitation). [245]

It the last part of chapter 2, we investigated whether a direct contact of *A. muciniphila* with the human colonic mucosa was safe and could trigger specific changes in the expression of certain genes. Though this study was meant as a pilot exploratory experiment and not in any way designed to assess any specific efficacy, we are confident that the data obtained are strong enough to demonstrate that *A. muciniphila* directly affects the expression of different genes. This confirms that this bacterium can dialogue directly with host cells. Although we have observed several alterations of the gene expression, either decreased or increased gene expression, we could not identify a direct effect of *A. muciniphila* on the expression of IL22 or IL17. This is likely explained by the fact that the subjects were healthy, and that no specific inflammation was induced. However, it is worth noting that among the genes induced by the exposition to *A. muciniphila,* several genes were identified that are under direct controlled of the TLR/MyD88 pathway. For instance, the expression of MARCO (macrophage receptor with collagenous structure) was among the most strongly induced genes. This marker is a scavenger receptor expressed on the cell surface of macrophages that mediates opsonin-independent phagocytosis. It has also been suggested that MARCO regulates the activation status of the immune system and can be involved in balancing a proper immune response to infection. This regulation could also be important in maintaining tolerance [258, 259]. Besides this important gene, we also identified a higher expression of DUOXA2 (duox activator) and DUOX2 (dual oxidase) that are involved in H2O2 production and also act as anti-microbial agents [280, 281]. Interestingly, *A. muciniphila* has two catalases to protect itself against oxidative stress.

This pilot study, although with its limitations, is the first demonstration that A. muciniphila directly dialogues with host cells and is sufficient to

modulate genes in the colon of humans, and clearly supports the need to design future clinical studies to identify whether both acute and chronic oral administration of *A. muciniphila* can replicate the effect. Moreover, it would be of interest to design a study to investigate whether a pretreatment with oral *A. muciniphila* can be associated with a lower incidence of post-operative comorbidities. However, such kind of studies will require additional safety tests. Finally, whether the use of live *A. muciniphila* is required should also be investigated. Indeed, recent data have shown that the pasteurization of *A. muciniphila* increases its efficacy in the context of obesity, diabetes and cardiometabolic disorders. In addition, the use of pasteurized *A. muciniphila* has been validated by the EFSA (European Food Safety Authority) as a novel food and can be safely used as a food supplement at a dose of 34 billion bacteria per day. We performed a first preliminary experiment using pasteurized *A. muciniphila* and our data were similar to the one observed with the live bacteria, indicating that this could be a feasible option for future clinical experiments.

In conclusion, in this thesis, we have developed a novel animal model which will be suitable for the study of colonic wound healing and the assessment of different modulators of the gut microbiota. We demonstrated that *A. muciniphila* has a protective effect against peritonitis-induced mortality via mechanisms associated with the innate immune system. Finally, we offered the first proof of concept that *A. muciniphila* can directly modulate the expression of genes in the human colon.

These results emphasize the need for further research into targeted approaches that can modify the gut microbiota in such a way that pathobionts are selectively inhibited, while beneficial bacteria are simultaneously preserved or even stimulated. Such a targeted treatment prior to surgery

would create the optimal intestinal ecosystem and minimize the risk of complications. To tap into the full potential of microbiota modulation it is important to develop tools and adequate protocols evaluating the clinical relevance of gut microbiota modulation to positively influence the wound healing. The data needed to achieve this goal must inevitably come from studies that focus not only on the composition but also on the specific management of the microbiota. Indeed, most of the data available today remains excessively descriptive, and we therefore recommend a better characterisation of the type of preparation and more detailed descriptions of the microbiota upon the onset of the disease. Deeper analyses of the microbiota are crucial to fully understand the specific interactions between the bacteria inhabiting the gut and their role in the complex wound-healing processes. While the role of the gut microbiota has been neglected for a long time, attention has recently risen for both the beneficial and pathological roles of the gut microbes in the healing of intestinal wounds.

In a later stage, this will hopefully help unlock the huge potential targeted use of new generation of probiotics, prebiotics and synbiotics represent.

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APPENDIX

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MISE AU POINT

La préparation colique en chirurgie colorectale

Bowel preparation prior to colorectal surgery

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MOTS CLÉS
Préparation du côlon ;
Infection du site opératoire (ISO) ;
Fistule anastomotique (FA)

Résumé La fistule anastomotique ainsi que les infections du site opératoire sont les causes principales de morbidité postopératoire après chirurgie colorectale avec une augmentation significative de la mortalité postopératoire. Essayer de prévenir leur apparition est, pour cette raison, la priorité de la préparation d'une chirurgie colorectale. Pour éviter leur survenue, la nécessité d'effectuer une préparation intestinale préopératoire et les différentes méthodes de réalisation de cette préparation, sont toujours débattues sans qu'il n'y ait de consensus international. Des études randomisées multicentriques qui comparent l'utilité d'une préparation mécanique du côlon associée ou non à l'administration d'antibiotiques oraux, viennent de débuter avec l'espoir que leurs résultats éclairciront au moins partiellement ce débat. Une nouvelle approche, pourrait consister à changer ou moduler en préopératoire le microbiote intestinal de telle façon qu'il favorise la guérison anastomotique et la récupération postopératoire en général.
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KEYWORDS
No bowel preparation (NBP);

Summary Anastomotic leak and surgical site infections are the most important causes of postoperative morbidity after colorectal surgery, with a significant increase in the post-surgical mortality. For this reason, the priority of the preparation for colorectal surgery is avoiding their

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Bowel preparation (MBP);
Oral antibiotic bowel preparation (OABP);
Mechanical and oral antibiotic bowel preparation (MOABP);
Surgical site infection. (SSI);
Anastomotic leakage (AL)

occurrence. The question whether a bowel preparation prior to surgery is necessary and what methods to use are still highly debated, and no consensus has been reached so far. In the past years, this controversy was revived following several studies with diverging results. A set of multi-center randomized studies comparing the effects of a mechanical bowel preparation, associated or not with oral antibiotic have just started, and their results may help shed light, at least partly, on this ongoing debate. On the other side, a new approach, outside the usual prospects, would be to try to change or modulate the intestinal microbiota prior to surgery so that it can eventually contribute to anastomotic healing and post-surgery recovery in general.
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Abréviations

PPC	pas de préparation du côlon
PMC	préparation mécanique du côlon
PCAO	préparation colique par des antibiotiques oraux
PMCAO	préparation mécanique du côlon associée à l'administration des antibiotiques oraux
ISO	infection du site opératoire
FA	fistule anastomotique
i.v.	intraveineux

Introduction

Le principe de la préparation colique avant chirurgie colorectale a été introduit, il y a plus de 50 ans. Il est devenu rapidement un dogme chirurgical. Initialement, il s'agissait d'une préparation mécanique du côlon par voie orale associée à l'administration d'antibiotiques oraux (PMCAO). Avec l'apparition des antibiotiques intraveineux (i.v.), est apparue une première scission entre des chirurgiens qui continuaient l'administration des antibiotiques oraux associées à l'administration préopératoire d'antibiotiques i.v. et d'autres, qui les avaient remplacés.

En effet, avec la prise de conscience de l'apparition de résistances aux antibiotiques et en prenant en compte les nombreux effets secondaires liés à l'administration d'une combinaison d'antibiotiques non-résorbables, la plupart des chirurgiens, surtout en Europe, ont décidé de remplacer l'administration préopératoire des antibiotiques oraux par une antibioprophylaxie i.v. administrée pour la première fois juste avant l'opération. C'est ainsi que la préparation la plus utilisée avant une chirurgie colorectale, était la préparation mécanique du côlon (PMC) sans administration d'antibiotiques oraux.

En 1997, le chirurgien danois Henrik Kehlet a introduit en chirurgie colorectale le concept de réhabilitation améliorée après chirurgie, ou « Fast-Track Surgery », ayant pour but de réduire au maximum l'impact du stress chirurgical sur l'homéostasie physiologique du patient. Même si à l'origine, le programme comprenait seulement quatre points (chirurgie mini-invasive, analgésie multimodale basée sur la péridurale thoracique, nutrition orale et mobilisation précoces), d'autres mesures sont venues s'ajouter afin d'optimiser la préservation de la fonction intestinale et de favoriser la mobilité du patient pour ramener l'état physiologique de celui-ci au niveau préopératoire le plus rapidement possible après une chirurgie majeure. C'est dans ce cadre que l'indication de la préparation colique mécanique a été mise en question. Plusieurs essais randomisés de grande envergure ont alors démontré l'absence d'avantages d'une préparation mécanique colique (PMC), surtout en préopératoire de chirurgie colique, raison pour laquelle elle a été considérée comme inutile pour toute chirurgie colique (non rectale) par les sociétés de réhabilitation améliorée après chirurgie (ERAS, GRACE).

Cependant, en 2015, trois articles publiés dans « Annals of Surgery », avec les résultats de plusieurs grandes séries rétrospectives issues du programme national d'amélioration de la qualité chirurgicale (ASC NSQIP) de *l'American College of Surgeons*, ont remis en question la tendance de ne plus préparer le côlon en préopératoire, et ont suggéré que la préparation mécanique du côlon associée à l'administration des antibiotiques oraux (PMCAO) diminuait le taux d'infection du site opératoire et la morbidité postopératoire en général.

Depuis lors, plusieurs méta-analyses ont comparé les résultats des études utilisant une préparation mécanique du côlon associée à l'administration d'antibiotiques oraux (PMCAO) avec la préparation mécanique colique (PMC) seule, en démontrant des avantages pour la PMCAO [1]. Par contre, il existe une seule étude randomisée multicentrique publiée cette année dans la revue *Lancet* qui compare la PMCAO avec l'absence de préparation (PPC) et qui montre que la PMCAO ne diminue pas le taux d'infection du site opératoire (ISO) ni n'augmente le taux de morbidité postopératoire générale [2].

La situation reste donc largement controversée avec une vision plutôt américaine en faveur de la PMCAO opposée à la vision européenne en faveur de la PPC, chacune étayée par de très bons arguments.

Le microbiote intestinal : « The new kid on the block »

Si on part du principe classique que le contenu intestinal représente un ensemble de bactéries pathogènes qui

potentiellement peuvent infecter le site opératoire et les sutures digestives, la logique de « vider et de désinfecter » le côlon avant une chirurgie colique est tout à fait évidente. Un côlon vide présente, en plus, l'avantage d'une manipulation plus facile, un atout de plus surtout dans le cadre de la chirurgie mini-invasive laparoscopique.

Durant la dernière décennie, les perspectives par rapport au microbiote intestinal ont changé : il est désormais considéré comme « nouvel organe » avec de nombreuses fonctions biologiques. Le microbiote intestinal n'est plus vu comme exclusivement un conglomérat des bactéries pathogènes responsables de l'apparition des complications postopératoires, mais de plus en plus d'études montrent le rôle favorable potentiel des certains composés du microbiote intestinal dans la cicatrisation intestinale et dans la récupération postopératoire en général [3].

Par ailleurs, vouloir écarter les quelques pathogènes en réalisant une purge associée à l'administration orale d'antibiotiques représente une vision trop « simpliste ». Si on prend en considération l'important rôle métabolique du microbiote, il faut forcément penser à l'interaction du microbiote avec l'organisme et à son rôle dans le maintien de l'homéostasie.

Le microbiote de l'organisme humain héberge toute une communauté de micro-organismes (bactéries, archées, levures et virus) tous présents au niveau des zones de contact avec le milieu extérieur. La surface de contact au niveau de la peau est d'environ 2 m^2, tandis que celle de l'intestin avec toutes ses villosités, est estimée à environ 250 à 400 m^2. Notre corps cohabite avec environ 100 000 milliards de bactéries (10^{14}), soit bien plus que nos propres cellules corporelles.

Sur le plan de l'information génétique véhiculée, le microbiote surpasse tout simplement le génome humain de plus de cent fois. En pratique, cela signifie que ces micro-organismes ont des fonctions métaboliques et biologiques que notre métabolisme humain ne peut pas accomplir. Ce qui se passe « génétiquement parlant » dans notre corps est en fait avant tout largement « microbien » et beaucoup d'échanges croisés ont lieu.

La répartition des bactéries le long du tube digestif n'est pas homogène. Le côlon est le lieu de vie de prédilection de nos bactéries intestinales. Il a été montré que l'estomac contient environ 10^1-10^3 bactéries par gramme de contenu gastrique tandis que côlon en contient 10^{10}-10^{11} par gramme et les fèces 10^{12}-10^{13} par gramme. Dans le tube digestif, on différencie également le microbiote associé à la muqueuse intestinale du microbiote intraluminal (les fèces), l'un étant proche de la couche de mucus intestinal et des cellules épithéliales et l'autre dans la lumière de l'intestin. Leur composition ainsi que leur fonction sont différentes et le microbiote associé à la muqueuse intestinale a probablement des fonctions métaboliques importantes pour nos cellules intestinales et sans aucun doute au-delà.

On sait aussi que le microbiote évolue en fonction de l'âge. Le microbiote des sujets âgés, qui représentent la majorité des patients ayant une pathologie colorectale, est différent de celui des adultes jeunes, avec une plus grande proportion de *Bacteroides species* et une abondance spécifique de différentes souches de *Clostridium*. Ceci pourrait contribuer à l'augmentation connue du risque de fistule anastomotique chez les personnes âgées.

Le microbiote est aussi très perturbé lors de la présence d'une pathologie comme le cancer par exemple et bien sûr aussi dans le cas des maladies inflammatoires.

Plusieurs études ont montré que certaines bactéries telles que *Fusobacterium nucleatum* ou encore *Bacteroides fragilis* et *Escherichia coli*, semblent émerger sous les traits de pathogènes et participer au développement du cancer colorectal à travers leurs propriétés inflammatoires et oncogéniques. *Fusobaterium* est souvent associée aux cancers colorectaux agressifs.

Différentes études ont tenté de déterminer quelles bactéries étaient associées aux fistules anastomotiques. En utilisant un modèle de rongeurs, l'équipe de Shogan a découvert des changements significatifs dans le microbiote associé aux tissus anastomotiques avec des différences minimales dans le microbiote luminal, notamment dans les selles. La différence la plus frappante était une augmentation de 500 fois de l'abondance relative d'entérocoques et une augmentation de 200 fois d'*Escherichia coli* et de *Shigella* à la suite d'une anastomose colorectale basse. Ensuite, ils ont aussi démontré les mécanismes par lesquels ces bactéries sont capables de dégrader une anastomose intestinale.

D'autre part, des efforts ont été faits afin d'identifier quelles bactéries pourraient favoriser la cicatrisation intestinale. Parmi les bactéries bénéfiques identifiées récemment, *Akkermansia muciniphila* est un candidat prometteur. Elle est l'une des espèces les plus abondantes dans le microbiote intestinal humain, avec une prévalence allant jusqu'à 5 % du total des bactéries. Elle est inversement associée à une inflammation de bas-grade et à des troubles métaboliques. Dans la littérature *Akkermansia muciniphila* est démontrée comme étant capable de renforcer la fonction de barrière intestinale via des mécanismes impliquant la production de mucus et de facteurs antimicrobiens, cependant son rôle direct sur la cicatrisation est inconnu.

À ce jour, il n'y a que peu d'études qui ont analysé et décrit l'impact d'une préparation mécanique colique (PMC) sur le microbiote intestinal, toutes avec un nombre limité de patients et des groupes assez hétérogènes (Tableau 1). Néanmoins, ces études ont permis d'identifier certaines tendances. La plupart des études ont confirmé le fait que la PMC avait un impact important sur la diversité du microbiote intestinal mais qu'elle ne décontaminait pas le tractus gastro-intestinal car la quantité de bactéries restait presque la même que lorsqu'elle n'était pas effectuée. Par contre, l'administration d'antibiotiques oraux provoque un effet immédiat et durable sur la diversité et la quantité microbienne, avec souvent à une récupération incomplète du microbiote. De plus, les solutions polyéthylène glycol (PEG) pour la PMC entraînent une perte importante du mucus superficiel au niveau de la muqueuse intestinale.

La preuve qu'une anastomose colorectale guérit sans complications sur un côlon non-préparé est donnée par l'exemple de la chirurgie traumatologique d'urgence. Une méta-analyse Cochrane a montré que la réalisation d'une anastomose primaire sans diversion et sans préparation est préférable en cas de lésion colique traumatique. Cependant, il faut savoir qu'en général, les traumatismes intestinaux sont observés lors de perforations par balle,

Tableau 1 Effet de la PMC sur la composition du microbiote.

	Année	N	Type	M vs S
Mai. V et al.	2006	5	–	S
Bucher P. at al	2006	50	PEG	M
Harrel L. et al.	2011	12	PEG	M
O'Brien C. at al	2013	15+5	PEG	S
Jalanka J. et al.	2015	23	PEG	S
Drago L. et al.	2016	10	PEG	S
Shobar et al.	2016	18	½ PEG/½ NaCl	S+M
Nagata et al.	2019	8+23	PEG	S
			Bachmann et al. Gut, 2017	

Type : type de préparation intestinale : solutions de polyéthylène glycol (PEG) ou solutions salines (NaCl). M vs S : bactéries associées au mucus versus bactéries à contenu luminal.

dans une population jeune et en bon état général, avec probablement un microbiote intestinal « en bonne santé ». Des résultats comparables ont été démontrés également pour les traumatismes du rectum pour lequels une suture ou une résection-anastomose sans dérivation et sans préparation était préférable.

Recommandations internationales divergentes

Les recommandations éditées par GRACE en 2014, ont été actualisées en ce qui concerne la préparation intestinale en 2018 [4]. Les recommandations de la société ERAS sont parues en 2018 tandis que celles de la société américaine de chirurgie colorectale ont été publiées début 2019.

L'American Society of Colon and Rectal Surgeons recommande, en 2019, une préparation préopératoire associant une purge (PMC) avec l'administration d'antibiotiques oraux (PCAO) pour toute intervention chirurgicale colique et rectale, (Fig. 1) [5], tandis que le Groupe Francophone de Réhabilitation Améliorée après Chirurgie (GRACE) [6] et la Société ERAS (Enhanced Recovery After Surgery) [7] ne recommandent pas de préparation (PPC) pour les interventions coliques.

Pour la chirurgie rectale, GRACE suggère que les preuves de la littérature ne suffisent pas pour pouvoir donner des recommandations fermes pour la réalisation d'une PMC. Le groupe ERAS affirme d'un autre côté que la PMC peut être utilisée pour la préparation de la chirurgie rectale. Il relève que certaines études montrent un avantage pour l'utilisation d'une PMCAO par rapport à une PMC. Par contre, il n'est pas recommandé de faire une PCAO exclusive.

Comme susmentionné, la société américaine de chirurgie colorectale recommande fortement l'utilisation d'une PMCAO pour toute intervention colique et rectale. Les recommandations insistent sur le fait que la PMC sans l'association d'une PCAO, n'est pas indiquée. Elles suggèrent également que l'usage de PCAO seule n'est pas conseillé. L'usage de lavement préopératoire est également déconseillé.

La société européenne de chirurgie colorectale (ESCP) n'a pas développé de recommandations concernant la préparation colique préopératoire.

Le taux de fistule anastomotique est toujours estimé entre 1 % et 19 % et entraîne des conséquences dramatiques, comme l'augmentation significative de la mortalité, la récidive en cas de cancer et associé à des coûts très importants.

Enfin, une enquête réalisée par l'ESCP en 2017 auprès des chirurgiens colorectaux a montré que les recommandations n'étaient pas respectées. Sur un échantillon de plus de 400 chirurgiens ayant répondu au questionnaire, plus de la moitié réalise toujours une préparation mécanique avant une chirurgie du côlon.

La controverse éternelle : préparer ou ne pas préparer et si oui, comment le faire ?

L'utilisation d'une antibioprophylaxie i.v. en préopératoire d'une chirurgie colorectale est communément acceptée. Mais le consensus s'arrête là. De nombreuses études et méta-analyses ont tenté de démontrer la supériorité d'une préparation par rapport à l'autre, mais la plupart des essais cliniques présentent des biais, n'atteignent pas la puissance statistique nécessaire et sont très hétérogènes, incluant par exemples des patients avec des pathologies différentes (oncologiques et non oncologiques), avec des localisations variées (côlon droit, transverse, gauche et rectum), etc. de plus, jusqu'à ce jour, aucune étude prospective randomisée en double aveugle n'a été réalisée avec les quatre conditions (PPC, PMC, PCAO, PMCAO).

Néanmoins, de nombreux essais ont comparé les différents types de préparations permettant quelques conclusions. Pendant les trois dernières décennies, différentes études prospectives suivies de revues systématiques et de méta-analyses ont comparé l'effet de la PMC à la PPC. Aucune revue systématique de la littérature n'a démontré un avantage pour les patients bénéficiant d'une PMC en préopératoire. Deux méta-analyses publiées en 2004 par Bucher et al. et Slim et al., ont même mis en évidence un taux significativement plus élevé de fistules anastomotiques chez les patients ayant une PMC en préopératoire. Cependant, les méta-analyses plus récentes n'ont plus montré de différence significative dans la survenue de fistule anastomotique et certaines d'entre-elles, avec un nombre plus important de patients, n'ont plus montré d'avantage significatif sur le taux d'infection du site opératoire (ISO) pour les patients

Figure 1. Synthèse des recommandations internationales.

PPC. Les méta-analyses récentes ne montrent aucune différence entre la PMC et la PPC en termes de FA, d'ISO, de ré-intervention chirurgicale ou de mortalité postopératoire.

Une seule étude randomisée a comparé la PMCAO à une PCAO. L'étude de Zmora et al. réalisée en 2003 chez 380 patients, n'a pas montré d'avantage pour un des deux groupes en ce qui concerne les ISO, FA, les complications non-chirurgicales et la mortalité globale. Une revue de littérature publiée en 2017 par Koullouros et al. concernant le rôle de la PCAO en chirurgie colorectale suggère une diminution significative de l'ISO suite à une PCAO, par contre pas d'influence sur l'apparition d'une fistule anastomotique. Deux autres méta-analyses de Chen et al. en 2016 et McSorley et al. en 2018 montrent des résultats similaires avec une amélioration de l'ISO suite à l'administration d'une PCAO.

Une seule étude randomisée multicentrique a comparé la PMCAO à la PPC. Elle vient d'être publiée cette année dans la revue *Lancet* et montre que la PMCAO ne diminue pas le taux d'infection du site opératoire (ISO) ni n'augmente le taux de morbidité postopératoire générale. Cependant, les résultats de cette étude sont à l'opposé des résultats de plusieurs grandes séries rétrospectives issues du programme national d'amélioration de la qualité chirurgicale (ASC NSQIP) de *l'American College of Surgeons* comme les résultats de l'analyse rétrospective de Scarorough et al. de 2015 sur 8500 de patientes, celle de l'analyse rétrospective de Kiran et al. de 2015 [8] sur 8400 de patients, celle de Garfinkle et al. de 2017 sur 40 000 patients et le Snapshot de l'ESCP [9] de 2017 sur 4000 de patients.

Conclusions et perspectives

Les résultats de larges études rétrospectives publiées en 2015 ont relancé le débat concernant l'utilité d'administrer des antibiotiques oraux dans la préparation intestinale préopératoire. Néanmoins, en raison de nombreuses limitations méthodologiques, cette question ne peut pas être tranchée en se basant sur ces seules études.

Pour cette raison, afin d'essayer d'élucider la question de la préparation intestinale, deux essais randomisés multicentriques en double aveugle à 4 bras (PPC, PMC PCAO, PMCAO) « COLONPREP » (pour la chirurgie colique) et « PREPACOL2 » (pour la chirurgie rectale) sont actuellement en cours.

Une autre alternative à la préparation mécanique ou à l'usage des antibiotiques oraux se profile à l'horizon, mais elle reste encore à explorer et elle pourrait réduire davantage encore le taux de fistules anastomotiques. Plutôt que d'utiliser une méthode « grossière » et « dévastatrice », comme c'est le cas quand lorsque nous utilisons une préparation intestinale associée aux antibiotiques oraux, une autre approche pour le futur serait de réaliser une préparation ciblée pour inhiber seulement les bactéries potentiellement « délétères » et « délétères » et de favoriser ainsi le développement de bactéries dites plutôt « bénéfiques » en « manipulant » en quelque sorte le microbiote.

Cette nouvelle approche consisterait donc à « changer » ou « moduler » en préopératoire le microbiote intestinal, de telle façon qu'il favorise la guérison anastomotique et la récupération postopératoire en général.

Si nous nous autorisons à rêver un peu, nous pourrions dire que la préparation colorectale du futur consistera pratiquement en un changement de régime préopératoire à court terme, associé à une administration de probiotiques, de prébiotiques, de minéraux ou de vitamines sur une courte période avant la chirurgie afin de réduire le poids des selles, d'augmenter la couche de mucus avec la barrière intestinale, de diminuer l'inflammation locale et générale et d'augmenter de manière significative le nombre de colonies bactériennes considérées comme globalement bénéfiques au détriment des souches nuisibles.

Déclaration de liens d'intérêts

R.B., D.L., C.R., M.V.H. et A.K. déclarent ne pas avoir de liens d'intérêts.

P.D.C. est détenteur de plusieurs brevets relatif à l'utilisation de *A. muciniphila* et de ses composants pour traiter l'obésité et les troubles associés. PDC est cofondateur de A-Mansia Biotech SA.

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