特別講演Ⅰ

Effect of a probiotic strain (*Lactobacillus farciminis*) treatment on colonic inflammation and hypersensitivity in rats: pathways of action involved

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I Introduction

The gut and immune system form a complex integrated structure that has evolved to provide effective digestion and defence against ingested toxins and pathogenic bacteria. The digestive tract is most frequently the object of functional and health claims and a large market already exists for gut-functionnal foods worldwide. Around 60% of functional foods principally pro- and prebiotics are targeted at the gut and the immune system (Arai et al., 2002). Despite evolution in the definition of probiotics, they can be generally defined as live microbial food ingredients that are beneficial to health (Salminen et al., 1998). A wide range of bacteria is used in foods but lactic acid producing bacteria such as lactobacilli and bifidobacteria, tend to predominate in the probiotic food sector (Mogensen et al., 2002).

A large body of literature deals with beneficial effects of different probiotic stains in the gastrointestinal tract physiopathology, providing promising perspectives for therapeutic use of probiotics. Among gastrointestinal pathologies revealing potential use of probiotics literature mention inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS). Indeed despite some negative data (Prantera et al., 2002; Kuisma et al., 2003) some strains may be considered as an alternative or adjunct therapy in IBD, since encouraging results have been obtained in IBD treatment (Malin et al., 1996 ; Kruis et al., 1997 ; Rembacken et al., 1999 ; Guslandi et al., 2000 ; Campieri et al., 1999; Gionchetti et al., 2000). Similarly, exogenous administration of several lactobacilli strains has been found to reduce the severity of experimental colitis in animals (Fabia et al., 1993; Mao et al., 1996; Madsen et al., 1999; Schultz et al., 2002). The mechanisms involved in the anti-inflammatory action of probiotics are based on some general properties of these protective bacteria, such as prevention of overgrowth of pathogenic microorganisms, reduction of bacterial translocation (Mao et al., 1996) or maintenance of mucosal barrier integrity (Fabia et al., 1993) and modulation of cytokine production (Schultz et al., 2002).

Irritable Bowel Syndrome (IBS) is a gastrointestinal disease with unknown etiology, characterized by abdominal pain, discomfort, altered bowl habit and is frequently associated with psychological distress (Whitehead et al., 1988; Thomson et al., 1999; Drossman et al., 1999; Collins et al., 1999). Manifestations such as altered gut motility and visceral

hypersensitivity are similar to those initiated by stressful stimuli in rats (Williams et al., 1988; Gue et al., 1997). Further, an increase of intestinal permeability has been observed in post-dysenteric IBS patients (Spiller et al., 2000). We have recently shown that stress increased colonic permeability was responsible of hypersensitivity to colorectal distension in stressed rats (Ait-Belgnaoui et al., 2005). Despite some clinical studies showing an improvement of IBS symptoms in humans (Niedzelin et al., 2001; Halpern et al., 1996; Kim et al., 2003), their role on the criterion visceral pain remains unknown.

A recent original approach for probiotic treatments and particularly in experimental colitis, entails the use of bacteria as a carrier of an anti-inflammatory compound which will be delivered in the gut lumen. In this way, Steidler et al. were able to reduce experimental colitis in mice genetically engineered to secrete the anti-inflammatory cytokine IL-10 (Steidler et al., 2000). Similarly, according to the possible anti-inflammatory action of nitric oxide (NO) delivered in the gut lumen, we have conducted studies using *Lactobacillus farciminis* (*L. farciminis*, CIP 103 136, Institut Pasteur, France), a strain known to release spontaneously NO *in vitro* (Wolf et al., 1990).

II *Lactobacillus farciminis* and experimental colitis

II 1 Role of exogenous NO

The first step of this study, was to evaluate the ability of *L farciminis* to release NO in the colonic lumen and to evaluate the effect of a chemical NO donor (sodium nitroprusside ; SNP) on trinitrobenzene sulphonic acid (TNBS)-induced colitis in rats. In a second part, we have determined the effect of *L. farciminis* treatment on TNBS-induced colitis as well as the role of NO released by this strain in this effect.

The ability of *L farcimins* to release NO in the colon was evaluated by measurement of NO in the colonic content by electron paramagnetic resonance.

SNP (1mg/kg/day) was infused continuously in the colonic lumen during four days post colitis and *L farciminis* (10^{12} CFU/day) was administered orally during 15 days before and four days after the induction of colitis. The role of exogenous NO delivered by SNP and *L farciminis* was evaluated using a NO scavenger, haemoglobin (200 mg/kg/day) infused in the colon.

The NO production measured *ex vivo* in the lumen of the colon was 1, 7-fold higher after oral treatment by *L farciminis* than in controls, underlying the ability of *L farciminis* to release NO *in vivo* conditions (Lamine et al., 2004 a).

Exogenous NO delivery by *L farciminis* and SNP in the colonic lumen exerts a protective effect against TNBS-colitis, since both treatments reduced macroscopic damage scores, myeloperoxidase and inducible nitric oxide synthase activities. This protective effect is linked to NO delivery by the chemical and biological NO donor since haemoglobin infusion reversed the anti-inflammatory effect of both treatments (Lamine et al., 2004 a).

II 2 Colonic responses

The reduction of TNBS-induced colitis severity by *L farciminis* was partially reversed by scavenging of NO by haemoglobin. Consequently, in a second study we aimed to determine whether the anti-inflammatory effect of *L farciminis* treatment partly due to the production of NO, was also associated with normalization of the colonic microflora, prevention of bacterial translocation, enhancement of barrier integrity and changes in cytokine profiles in rats.

L farciminis (10^{12} CFU/day) was administered orally during 15 days before and four days after the induction of colitis and the following parameters were evaluated: cytokine mucosal levels, bacterial profile in colonic content and mucosa, bacterial translocation and colonic paracellular permeability.

In basal conditions (absence of inflammation) *L farciminis* treatment reduced colonic paracellular permeability and increased the IL-10 level in the colonic wall. Further, *L. farciminis* was identified in faecal samples, showing the ability to survive through the gastrointestinal tract. It was also identified in colonic content and colonic mucosa from the 5^{th} to the 19^{th} days of treatment, indicating that it was able to persist into the gut (Lamine et al., 2004 b). Colitic rats presented increased bacterial translocation, colonic paracellular permeability, and IL-1β mucosal level and decreased Il-10 mucosal level (Lamine et al., 2004 b). Moreover in colitic rats the bacterial profile of colonic content and mucosa was modified. We observed a 12-fold and 15-fold increase in the number of total aerobic bacterial and total Gram-negative bacterial levels respectively in faeces. Concerning the number of bacteria adhering to or invading the colonic tissue, we also observed a significant increase in total aerobic and Gram-negative aerobic levels (24-fold) and total anaerobic Gram-negative bacteria counts (16-fold). All alterations induced by TNBS administration were abolished or significantly reduced by L. farciminis treatment (Lamine et al., 2004 b).

II 3 Conclusion

Taken together the results of these studies, highlight that *L farciminis* can be considered as a promising treatment for reducing colonic inflammation. Its anti-inflammatory action seems to

depend upon the NO release in the lumen and among the possible mechanisms of action linked to the NO-dependent pathway an indirect inhibition of inducible NO synthase activity can be proposed, and the subsequent reduction of NO tissue concentration may be important. Further, *L farciminis* shares the general mechanisms involved in the beneficial effects of probiotic treatment. It is likely that *L farciminis* exerts an anti-inflammatory effect on TNBS-induced colitis, through the association of different pathways such as ability to normalize colonic microflora, to prevent bacterial translocation, to enhance barrier integrity, to decrease mucosal level of pro-inflammatory cytokines and to release NO in the colonic lumen.

III Lactobacillus farciminis and acute stress

III 1 *Lactobacillus farcimins* and stress-induced hypersensitivity

In this study we aimed to evaluate the effects of *L. farciminis* treatment in a non inflammatory model of visceral hypersensitivity. Previous studies have shown that in rats, acute restraint stress is known to enhance abdominal response to rectal distension, particularly in females (Gue et al. 1997; Bradesi et al., 2002). Since the effect of probiotic treatments on visceral sensitivity has not been investigated yet, we evaluated the effect of *L. farciminis* treatment on stress-induced visceral hyperalgesia in rats.

L. farciminis (10^{12} CFU/day) was administered orally during 15 days before stress application lasting 2 hours. The stress used in this study, was a partial restraint stress (PRS) which is a mild and non-ulcerogenic stressor. The colorectal distension was performed by insertion of the balloon in the rectum. Isobaric distensions of the colon were performed by connecting the balloon to a computerized barostat and were performed from 0 to 60 mmHg (increment 15 mm Hg), each distension step lasting 5 min. Animals were also equipped with NiCr implanted into the abdominal external oblique muscle, in order to register abdominal contractions by electromyography.

Partial restraint stress increased the number of abdominal contractions for pressures of colorectal distension applied corresponding to 15-60 mmHg compared to controls. *L. farciminis* treatment prevented the increase of the number of abdominal contractions induced by stress, whatever the pressure of colorectal distension.

III 2 *Lactobacillus farciminis* and stress-induced increase of colonic paracellular permeability

In animals repeated stress increases colonic paracellular permeability through a contraction of cytoskeleton linked to activation of myosine light chain kinase (MLCK) of epithelial cells

(Ferrier et al., 2003). Further, we have recently show that acute stress increased colonic paracellular permeability is responsible of hypersensitivity to colorectal distension in stressed rats (Ait-Belgnaoui et al., 2004). Consequently, the aim of this study was to evaluate the effect of *L. farciminis* treatment on stress-induced increase of colonic paracellular permeability.

The protocol previously described was used. At the end of PRS session, evaluation of colonic paracellular permeability was performed using ^{51}Cr-ethylene diamine tetra acetic acid as a marker of paracellular permeation of tight junction, administered intracolonically. Urines were collected during 24h and radioactivity was measured. Permeability to ^{51}Cr-EDTA was expressed as the percentage of the total radioactivity administered.

Partial restraint stress increased colonic paracellular permeability compared to controls and *L. farciminis* treatment prevented the stress-induced increase of colonic paracellular permeability.

III 3 Conclusion

Taking together the results of these studies we can speculate that *L farciminis* treatment prevents increase of colonic paracellular permeability induced by stress (probably trough an action on epithelial cells cytoskeleton) which in turn leads to a decrease of visceral hypersensitivity induced by stress. However, the mechanism of action of *L farciminis* on the epithelial cell cytoskeleton needs further investigations. Therefore, taking into account the place of stress in the genesis of functional bowel disorders, as well as the cause effect relationship between changes in colonic permeability and visceral hyperalgesia induced by stress, we can speculate that *L. farciminis* may be a promising agent for the treatment of digestive pathologies such as IBS.

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ラットにおける大腸炎と過敏性腸症候群に対するプロバイオティクス菌株(*Lactobacillus farciminis*)の効果:　作用機構に関して

I 緒言

腸管と免疫システムはひとつの複合体構造を形成している。この構造は摂取した毒物を効率よく解毒し、病原菌による感染を防御するために進化してきたものである。消化管は機能性クレームとヘルスクレームの対象としてもっとも多く取り上げられ、腸・機能性食品は世界中に大きな市場を形成している。機能性食品のおよそ 60%（その多くはプレ/プロバイオティクスであるが）は、腸や免疫システムをターゲットとしたものである(Arai et al., 2002)。プロバイオティクスという言葉の定義はさまざまに変わってきているが、一般的には健康に有用な効果をもたらす生きた微生物の食品添加物と定義できるであろう(Salminen et al., 1998)。

実に多様な菌種の細菌が食品には用いられている。この中で Lactobacilli や Bifidobacteria のような乳酸を産生する細菌はプロバイオティクス食品の分野で主に用いられている(Mogensen et. al., 2002)。種々のプロバイオティクス菌株に関して、多くの文献が消化管への生理学的、病理学的な有用効果を論じており、プロバイオティクスを治療目的に使用することへの明るい見解を示している。また多くの文献は、プロバイオティクス活用の可能性が示されている消化器疾病として炎症性大腸疾患(IBD)や過敏性腸症候群(IBS)を挙げている。中には IBD の治療のためプロバイオティクスを応用することを否定する報告もあるものの(Prantera et al., 2002; Kuisma et al., 2003)、肯定的な報告もあり(Malin et al., 1999; Kruis et al., 1997; Rembacken et al., 1999; Guslandi et al., 2000; Campieri et al ., 1999; Gionchetti et al., 1996)、いくつかのプロバイオティクス菌株は大腸炎の代替療法または補助療法として使用可能な菌株となりうることを支持する結果が得られている。同様に、いくつかの Lactobacilli 菌株の投与が動物における実験大腸炎の重篤度を軽減することがわかってきた。(Fabia et al., 1993; Mao et al., 1996; Madsen et al., 1999; Schultz et al., 2002)プロバイオティクスにおけるこうした抗炎症作用の作用機作は、病原細菌の過剰増殖の抑制、バクテリアルトランスロケーションの抑制(Mao et al., 1996)、粘膜防御の維持(Fabia et al., 1993)、サイトカインの産生調節など(Schultz et al., 2002)、プロバイオティクスが一般的に有する性質に基づいたものである。

過敏性腸症候群(IBS)は原因不明の消化管疾病で、腹痛・不快感・便通異常を特徴とし、しばしば精神的な落ち込みを伴う(Whitehead et al., 1988; Thomson et al., 1999; Drossman et al., 1999; Collins et al., 1999)。これら消化管運動の変調や内臓過敏症といった症状は、ラットにストレス刺激を与えたときに引き起こされる症状と似ていた(Williams et al., 1988; Gue et al., 1997)。さらに赤痢罹患後の IBS 患者の腸においては、透過性の増大が観察されている(Spiller et al., 2000)。我々は最近、ストレスによって惹起される腸透

過性の増大が、ラットにおける結腸直腸拡張をもたらす過敏症の原因となる可能性があることを示した(Ait-Belgnaoui et al., 2005)。いくつかの臨床試験ではヒトにおける IBS の症状改善が示されているものの(Niedzelin et al., 2001; Halpern et al., 1996; Kim et al., 2003)、腹痛の改善効果に対してどのような機作で効くのか未だ不明である。

プロバイオティクス処方に関する近年のオリジナルアプローチでは、特に実験大腸炎を中心として、胃腸の管腔内へ抗炎症性物質を運ぶ運搬者として細菌を利用しようとしている。このような考えの下、Steidler らは、抗炎症性のサイトカインである IL-10 を分泌するよう遺伝子工学的手法を（プロバイオティクスに：訳者挿入）施すことによって、マウスの実験大腸炎を軽減させることに成功した(Steidler et al., 2000)。同様な考えから我々は、腸管内腔へ送られた一酸化窒素(NO)によって抗炎症効果が発揮されることが可能かどうかを検証するため、*in vitro* の試験で NO を産生することが明らかになっている(Wolf et al., 1990) *Lactobacillus farciminis* (CIP 103 136, Institut Pasteur, France)を用いて実験を行った。

II *Lactobacillus farminis* と大腸炎モデル

II 1 外因性 NO の役割

実験の第一段階として、*L. farminis* が大腸管腔内に NO を放出する能力と、次いで、トリニトロベンゼンスルホン酸(TNBS)により大腸炎を誘導したラットにおいて、NO の化学的供与体であるナトリウムニトロプルシド(SNP)の投与効果を評価した。第二段階として、TNBS で大腸炎を誘導されたラットにおいて、*L. farminis* による治療効果を確認するとともに、この株が産生する NO による治療効果を明らかにした。

結腸における *L. farciminis* の NO 産生能は、結腸中の NO 量を電子磁気共鳴法で測定することにより評価した。治癒効果を評価するために（訳者挿入）、SNP(1 mg/kg/day)は大腸炎誘導後、結腸管腔内に 4 日間注入され、*L. farciminis* (10^{12} CFU/day)は大腸炎の誘導前 15 日間および誘導後の 4 日間、経口的に投与された。

SNP や *L. farciminis* によってもたらされた外因性 NO の役割は、NO を捕捉するヘモグロビン(200 mg/kg/day)を結腸に注入することにより評価した。

ex vivo で測定された結腸ルーメンの NO 産生量は、対照群と比較し *L. farcminis* 経口投与群で 1.7 倍に上昇したことから、*L. farciminis* が *in vivo* において NO を放出する能力があることが示唆された(Lamine et al., 2004 a)。SNP や *L. farciminis* によって結腸内腔に運搬される外因性 NO は、TNBS によって誘導される大腸炎に対して保護効果を示した。即ち SNP や *L. farciminis* の投与は、外観によるダメージスコアの軽減やミエロペルオキシダーゼの減少、誘導性の NO 合成酵素の活性抑制をひきおこした。ヘモグロビンを注入すると双方（化学的あるいは生物的：訳者加）の NO 供与体による抗炎症効果が低減することからも、化学的あるいは生物的 NO 供与体による NO の供与が、保護的な効果と関連

があることが分かる(Lamine et al., 2004 a)。

II 2 結腸での反応

L. farciminis による TNBS 誘導大腸炎の症状軽減は、ヘモグロビンにより NO を捕捉することで部分的に抑制された。そこで、次の実験において我々は、*L. farciminis* による抗炎症効果は、部分的に NO の産生に依っているのかについて検討し、また同効果は、ラットの結腸における菌叢の正常化、バクテリアルトランスロケーションの阻害、腸管のバリア機能の亢進、サイトカイン-プロファイルの変化といったことにも関与しているかについて検討した。*L. farciminis*(10^{12} CFU/day)を大腸炎誘導前 15 日間、誘導後 4 日間、経口的に投与し、以下の項目について測定した：結腸粘膜中のサイトカインレベル、結腸内容物および結腸粘膜における細菌叢、バクテリアルトランスロケーション、結腸細胞透過性。

炎症が見られない正常な状態では、*L. farciminis* 投与により結腸の細胞透過性が低下し、結腸壁における IL-10 産生量が増大した。さらに糞便中に *L. farciminis* が検出されたことから、*L. farciminis* が胃や腸を生きて通過できることが示された。また、投与開始から 5-19 日目の結腸内容物および結腸粘膜からも *L. farciminis* が検出されたことから、*L. farciminis* が腸内に生息できることが示唆された(Lamine et al., 2004 b)。大腸炎のラットでは、バクテリアルトランスロケーションの進行、結腸細胞膜透過性の増大、粘膜中 IL-1β の増大と IL-10 減少が見られた(Lamine et al., 2004 b)。さらに、大腸炎のラットにおいては結腸内容物および粘膜で細菌叢の変化がみられ、糞便中の総好気性細菌数は 12 倍に、総グラム陰性細菌数は 15 倍にまで増加していた。結腸組織に接着または侵入している細菌数についても測定すると、総好気性グラム陰性細菌は 24 倍に、総嫌気性グラム陰性細菌は 16 倍と顕著に増加していた。TNBS の投与によって生じる変化は全て、*L. farciminis* の投与により完全にあるいは顕著に打ち消されていた(Lamine et al., 2004 b)。

II 3 結論

これらの実験結果を考え合わせると、*L. farciminis* 投与による大腸炎症軽減治療は有望であると考えることが出来る。そしてこの抗炎症効果は腸内腔への NO の放出と関係があると考えられる。また、その抗炎症効果は、提案出来るとすれば、誘導性の NO 合成酵素の活性が間接的に阻害を受けるような NO dependent pathway にリンクしているというような機作に依っているように見える。これ（酵素活性阻害：訳者挿入）によって生ずる組織中の NO 濃度低下が（病体惹起に：訳者挿入）重要であるのかもしれない。さらに *L. farciminis* は通常のプロバイオティクス菌株に期待される機能を有している。*L. farciminis* は、結腸細菌叢の正常化、バクテリアルトランスロケーションの阻害、腸管のバリア機能の亢進、粘膜での炎症性サイトカインの減少、結腸内腔への NO 放出といったさまざま経

路を通して、TNBS 腸炎に対して抗炎症効果を発揮すると考えられる。

III *L. farciminis* と急性ストレス

III 1 *L. farciminis* とストレス誘導性過敏症

我々は本研究で炎症のないラットを用いて、*L. farciminis* の内臓過敏症に対する投与効果について評価した。これまでの研究により、ラットに急激なストレスを与えると、特に雌において直腸拡張を引き起こすことが分かっている(Gue et al., 1997; Bradesi et al., 2002)。内臓の感受性に対するプロバイオティクスの効果についてはこれまで調べられたことがなかったため、我々はラットのストレス誘導性内臓痛覚過敏に対する *L. farciminis* の投与効果を評価した。

2 時間に及ぶストレスを与える前の 15 日間 *L. farciminis*(10^{12} cfu/day)を経口的に投与した。本研究でのストレスとは、穏やかな部分緊張ストレス(PRS)で、潰瘍誘発性のストレスではない。結腸直腸拡張の状態は直腸に風船を挿入することによって作り出した。風船をコンピュータ制御の自動等圧器に接続し、5 分毎に 15mmHg ずつ増圧し、段階的に 0 mmHg から 60mmHg へと等圧的に結腸を拡張させた。また、腹部の収縮を electromyography で記録する目的で、この実験動物の腹部斜筋に NiCr を埋め込んだ。

15-60 mmHg の結腸直腸拡張による PRS は、コントロールと比較して腹部の収縮回数を増大した。結腸直腸拡張の圧力の大きさに関わらず、*L. farciminis* 投与処置によってストレス誘発性の腹部収縮の増大が抑制された。

III 2 *L. farciminis* とストレス誘発性結腸細胞透過性増加

動物に繰り返しストレスを与えると、上皮細胞のミオシン L 鎖キナーゼ(MLCK)の活性化に伴う細胞骨格の収縮によって結腸細胞透過性が増大する(Ferrier et al., 2003)。また我々は最近、ストレスを与えられたラットでは、急性ストレスによって結腸細胞透過性が増大すると、過敏症となり結腸直腸拡張を起こしてしまうことを示した(Ait-Belgnaoui et al., 2004)。そこで、この研究の目的はストレス誘導性の結腸細胞透過性に及ぼす *L.farciminis* 投与の影響を明らかにすることとした。

実験プロトコールは前述の方法を用いた。部分緊張ストレス処理の終了時に、タイトジャンクションの細胞透過性のマーカーとして結腸内に ^{51}Cr-EDTA を投与し、結腸細胞透過性を調べた。24 時間にわたって尿を採取し、放射性物質の量を測定した。^{51}Cr-EDTA の透過性は、投与された全放射性物質に対するパーセンテージで表した。

部分緊張ストレスはコントロールと比較して結腸細胞透過性を増大させ、*L. farciminis* を投与することによってストレス誘発性の結腸細胞透過性の増大が抑制された。

III 3 結論

これらの結果を考え合わせるとおそらくは、*L. farciminis* 投与はストレスによって誘導される結腸細胞透過性の増加を妨げ（たぶん上皮細胞の細胞骨格に作用することによって)、その結果ストレスによって引き起こされる内蔵過敏症を和らげると推測することができる。しかし、*L. farciminis* の上皮細胞の細胞骨格に及ぼす作用メカニズムに関してはさらに検討が必要である。従って、腸の機能障害を引き起こすストレス部位および結腸透過性とストレス誘発性内臓過敏症との因果関係を考慮すれば、*L. farciminis* の処方は IBS のような消化器病状において有効な治療手段となりうると推測される。

IV 引用文献

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