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Genetically engineered *Lactobacillus paracasei* rescues colonic angiotensin converting enzyme 2 (ACE2) and attenuates hypertension in female *Ace2* knock out rats

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Abstract

Engineered gut microbiota represents a new frontier in medicine, in part serving as a vehicle for the delivery of therapeutic biologics to treat a range of host conditions. The gut microbiota plays a significant role in blood pressure regulation; thus, manipulation of gut microbiota is a promising avenue for hypertension treatment. In this study, we tested the potential of *Lactobacillus paracasei*, genetically engineered to produce and deliver human angiotensin converting enzyme 2 (Lacto-hACE2), to regulate blood pressure in a rat model of hypertension with genetic ablation of endogenous *Ace2 (Ace2*$^{-/-}$ and *Ace2*$^{-/y}$*)*. Our findings reveal a sex-specific reduction in blood pressure in female (*Ace2*$^{-/-}$) but not male (*Ace2*$^{-/y}$) rats following colonization with the Lacto-hACE2. This beneficial effect of lowering blood pressure was aligned with a specific reduction in colonic angiotensin II, but not renal angiotensin II, suggesting the importance of colonic *Ace2* in the regulation of blood pressure. We conclude that this approach of targeting the colon with engineered bacteria for delivery of ACE2 represents a promising new paradigm in the development of antihypertensive therapeutics.

Graphical Abstract

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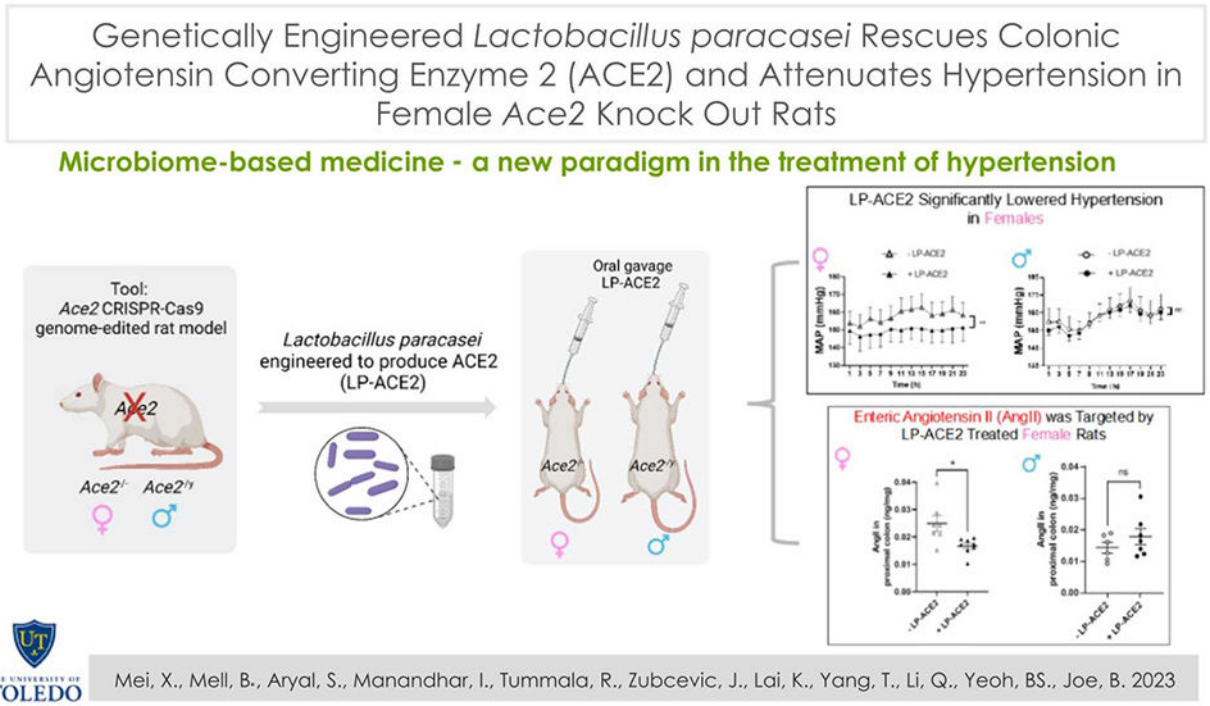
Declaration of Competing Interest

The authors declare no competing interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2023.106920.



Keywords

Bacterial engineering, human ACE2; *Lactobacillus paracasei* ; $Ace2^{-/-}$; Hypertension; Sex-specific

1. Introduction

Hypertension is the leading cause of death globally, affecting upwards of one in every four men and one in every five women [1,2]. It affects more than 1 billion adults worldwide, and is projected to rise to 1.54–1.58 billion adults by 2025 [3]. The renin angiotensin system (RAS) is a well-established mechanism for regulating blood pressure (BP), targeted by many antihypertensive therapies to reduce the effects of angiotensin II (AngII).

Components of the RAS are found throughout the body, including in the gastrointestinal (GI) tract [4]. Enteric RAS regulates an array of GI physiologic functions, including glucose absorption and inflammation [4-6]. Recently, it has been increasingly recognized that the gut is an important, previously neglected organ that contributes to BP regulation [7-9], and the role of the gut and its resident gut microbiota in hypertension has been shown by us and others [10-12].

Supplementation with probiotics as hypertension treatment has revealed inconsistent results [13-15]. Therefore, genetically modified probiotics have a great potential as next-generation therapeutics [16, 17]. *Lactobacillus*, a common microbe in the healthy gut microbiota [18] is frequently used as a health-promoting probiotic in fermented foods [19-21], and is reportedly reduced in abundance in hypertensive individuals [22]. This makes *Lactobacillus*

an ideal vehicle probiotic for delivery of engineered biologics. Indeed, such efforts are already underway for a variety of conditions including diabetic retinopathy [23-26], cancer [27,28], infection [29,30], and inflammatory bowel disease [31,32], but to our knowledge, recombinant *Lactobacillus* expressing human ACE2 has not been tested for hypertension. Since ACE2 is a positive modulator of BP that regulates AngII levels [33], we hypothesized that rescue of *Ace2* by colonization with *Lactobacillus paracasei* engineered to express human ACE2 will ameliorate hypertension in rodents with genetic ablation of endogenous *Ace2*.

To test this hypothesis, we generated a CRISPR/Cas9 gene-edited rat with targeted ablation of the endogenous *Ace2* locus on the genetic background of the Dahl salt-sensitive (S) rat. Engineered *Lactobacillus* expressing human ACE2 (Lacto-hACE2) was administered to address if the human ACE2 can be successfully delivered by the *Lactobacillus* carrier to rescue hypertension. We observed a significant reduction in BP following colonization with Lacto-hACE2 but only in the females $Ace2^{-/-}$ and not male $Ace2^{-/y}$ rats. This was associated with reduction in colonic but not renal AngII, suggesting a role for colonic RAS in sex-specific regulation of BP. This is the first report demonstrating the utility of an engineered microbes in antihypertensive therapeutics.

2. Materials and methods

2.1. Animals and housing conditions

The University of Toledo's Institutional Animal Care and Use Committee approved all animal research protocols (reference numbers: 104573–19 for breeding and 108390–08 for experiments). Experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the results were reported per the Animal Research: Reporting of In Vivo Experiments Guidelines. All rats were housed in cages with *Carefresh* paper bedding in the Department of Laboratory Animal Resources at the University of Toledo. The animal rooms were maintained at 70 ± 2°F. The humidity in the animal rooms was maintained at 50 ± 20 %.

2.2. Generation of Ace2 Knock Out ($rAce2^{-/-}$ and $rAce2^{-/y}$) rat model by CRISPR/Cas9 gene editing

Guide RNA (gRNA, sequence TTTTATGAAGAACAGTCCAAG, University of Michigan Transgenic core services) was designed to target the *Ace2* locus of intron 1 and exon 2 with no off-target sites. Oocyte microinjections were conducted at the University of Michigan Transgenic Animal Model Core (Ann Arbor, MI). A mixture of the gRNA (2.5 ng/μl) and Cas9 mRNA (5 ng/μl) was injected into one-cell stage Dahl S rat embryos. Microinjected embryos were implanted into 6 pseudo-pregnant Sprague Dawley female rats. Tail tip biopsies of newborn pups were collected and genomic DNA was extracted. Genotyping was performed using the following primers: Forward (5'TTGGTTTCTTGCCATGCAGC3') and Reverse (5'GACGCTTGATGGTCGCATTC3'). A potential founder rat was identified by the shorter size of DNA fragment than the non-founder (NF). The identified founder was backcrossed to the S rat, and their pups were intercrossed to obtain homozygosity of the disrupted *Ace2* allele. PCR products obtained from the homozygotes were shipped

to Eurofins MWG Operon (https://www.eurofinsgenomics.com/en/home.aspx), for DNA sequencing (Sequencher 5.4.6.). The heterozygotes of females were excluded for potential residual effect of endogenous *Ace2*; the homozygotes with fully disrupted *Ace2* locus were used for subsequent phenotypic studies. The female r*Ace2* KO rat was designated as r$Ace2^{-/-}$, while the male KO rat was labeled as r$Ace2^{-/y}$. The control rat used in this study was the NF rats which underwent the microinjection procedure for genome editing, but retained *Ace2*.

2.3. Genetic engineering of recombinant Lactobacillus paracasei for delivery of human Ace2 in the GI tract

The *Lactobacillus paracasei* (LP), which is used as a probiotic and also a gut microbe were engineered to produce the full length human ACE2 as previously described [23]. The construct was created to express and secrete the human ACE2 protein, and given via gavage into the intestinal lumen, as described in Prasad et al.[24] (Lacto-hACE2). The humanized ACE2 protein was fused with a carrier protein cleaved upon absorption to facilitate transport across the gut epithelium.

2.4. BP Measurements by radiotelemetry

Four groups of age- and sex- matched NF, r$Ace2^{-/-}$ and r$Ace2^{-/y}$ rats were weaned at 4 weeks onto a low salt diet (0.3 % NaCl, Harlan Teklad, TD 7034) for 11–12 weeks. Radiotelemetry transmitters (DSI, https://www.datasci.com) were implanted in all rats for continuous measurements of BP. A separate group of age-and sex-matched r$Ace2^{-/-}$ and r$Ace2^{-/y}$ were gavaged with either Lacto-hACE2 or Lacto-Control, starting from 12 to 13 weeks of age, three times per week for 3 weeks (600 μl, 10^{10} CFU/rat). BP (systolic, diastolic, and mean arterial pressure) and heart rate were recorded in all rats continuously for 21/23 h once every week using radiotelemetry and the BP and heart rate data on week 3 were analyzed with Ponemah (DSI). All values were collected continuously and averaged every 2 h.

2.5. Reverse transcription-polymerase chain reaction

At endpoint, all rats were euthanized by excess carbon dioxide exposure and proximal colon and kidneys were harvested for biochemical analyses. RNA was extracted from the proximal colon of animals using the TRIzol method as described [34]. Reverse transcription-polymerase chain reaction was performed to obtain cDNA using the Superscript III kit (Invitrogen). The resultant cDNA was diluted, and real-time polymerase chain reaction analysis using the SYBR Green master mix (Applied Biosystems) was done for transcripts of glucose transportation, glucose metabolism, anti-inflammatory genes, and sodium transportation. The representative transcripts evaluated including: *Glut1* (glucose transporter 1), *Eno1* (enolase 1), *Ldha* (lactate dehydrogenase A), *Pgam1* (phosphoglycerate mutase 1); *Il6 (interleukin 6), Il10 ((interleukin 10), Il17a ((interleukin 17A), Scnn1a (sodium channel epithelial 1 subunit alpha)*. Expression of these genes was quantitated for their relative expression to *Gapdh* using the $2^{-\Delta Ct}$ method [35]. All primer sequences are listed in Supplemental Table S1.

2.6. ACE2 activity, Ang II and glucose assays

To confirm that *Ace2* gene disruption is also functional, ACE2 Activity Assay Kit (#GR3438313–1, Abcam, ab273297) and AngII Assay Kit (#EK-002–12, Phoenix) were used to measure ACE2 activity in the kidney and proximal colon of NF and KO rats, as well as the AngII protein levels in the kidney and proximal colon of Lacto-Control-treated and Lacto-hACE2-treated *Ace2* KO rats.

2.7. Statistical analyses

Graph Pad Prism version 9.1.1 was used for statistical analyses. Unpaired t-test was used to compare ACE2 activity, AngII levels, and the relative genes expression levels between Lacto-Control and Lacto-hACE2-treated *Ace2* KO rats for both sexes.Two-way ANOVA (Analysis of Variance) with Fisher's LSD (Least Significant Difference) test was used for BP and heart rate comparisons between NF and KO, as well as Lacto-Control-treated and Lacto-hACE2-treated *Ace2* KO rats. Statistically significant values were represented as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****). All the figures with scattered dots are expressed as Mean ± SEM.

3. Results

3.1. CRISPR/Cas9-based genomic disruption of Ace2 in the hypertensive S rat reduced renal and colonic Ace2 activity

To detect Ace2 disruption, *Ace2* primers were designed against the CRISPR-edited area on the *Ace2* locus. Fig. 1A shows a full band in NF and a shorter band in the KO rats, confirming deletion of the *Ace2* locus. In Fig. 1B, our targeted gene-editing approach resulted in a 935 base pairs deletion within the *Ace2* locus (partial intron 1 and exon 2) in one of the pups, which was designated as the KO rat (the founder rat). As a result of *Ace2* locus deletion, both female and male r*Ace2* KO rats demonstrated significantly lower renal and colonic ACE2 activity when compared to the NF rats (Fig. 1C).

3.2. Disruption of Ace2 increases BP in male and female rats

As is shown in Fig. 2, compared with the NF rats, both female and male *Ace2* KO rats showed significantly higher systolic BP (NF female, SBP: 165 ± 2 mmHg; r$Ace2^{-/-}$ female, 208 ± 13 mmHg, $p < 0.0001$; NF male, 145 ± 3 mmHg; r$Ace2^{-/y}$ male, 168 ± 6 mmHg, $p < 0.0001$), diastolic BP (NF female, DBP: 115 ± 2 mmHg; r$Ace2^{-/-}$ female, 146 ± 9 mmHg, $p < 0.0001$; NF male, 101 ± 2 mmHg; r$Ace2^{-/y}$ male, 114 ± 6 mmHg, $p < 0.0001$) and mean arterial pressure (NF female, MAP: 132 ± 2 mmHg; r$Ace2^{-/-}$ female, 167 ± 10 mmHg, $p < 0.0001$; NF male, 116 ± 2 mmHg; r$Ace2^{-/y}$ male, 132 ± 6 mmHg, $p < 0.0001$).

3.3. Oral delivery of hACE2 via engineered Lactobacillus paracasei reduces BP in female but not male Ace2 KO rats

Male and female *Ace2* KO rats were treated with genetically-engineered *Lactobacillus paracasei* to deliver human ACE2 (Lacto-hACE2). As shown in Fig. 3, the r$Ace2^{-/-}$ rats gavaged with Lacto-hACE2 for 3 weeks significantly lowered diastolic (DBP, Lacto-Control, 145 ± 8 mmHg; Lacto-hACE2, 133 ± 9 mmHg, $p < 0.001$) and mean arterial pressure (MAP,

Lacto-Control, 158 ± 7 mmHg; Lacto-hACE2, 150 ± 7 mmHg, $p < 0.01$) when compared to the control group gavaged with the wild-type *Lactobacillus paracasei* (Lacto-Control). We observed no significant effect of either bacteria on SBP in female r*Ace2*$^{-/-}$ rats. Moreover, we observed no significant differences in systolic, diastolic, or mean arterial pressures in male r*Ace2*$^{-/y}$ rats treated with either Lacto-hACE2 or Lacto-Control, suggesting sex-specific effects of Lacto-hACE2.

3.4. Lacto-hACE2 reduces colonic but not renal AngII levels

Considering the role of ACE2 in lower BP by metabolism of AngII, we investigated AngII protein levels in the kidney and colon in *Ace2* KO rats treated with Lacto-Control and Lacto-hACE2. As shown in Fig. 4A, we observed significantly reduced AngII levels in the colon (Lacto-Control, 0.025 ± 0.003 ng/mg; Lacto-hACE2, 0.0167 ± 0.001 ng/mg, $p < 0.05$) but not the kidney (Fig. 4B) of female r*Ace2*$^{-/-}$ rats treated with Lacto-hACE2 compared to Lacto-Control. This effect was not observed in the male r*Ace2*$^{-/y}$ rats. These data suggest a role for colonic AngII in regulation of BP in female rats only.

3.5. Administration of Lacto-hACE2 decreased relative expression levels of colonic glucose transport- and metabolism-related transcripts in female Ace2 KO rats

Considering the role of AngII in inflammation, glucose and sodium handling [36-40], we tested the relative expression levels of several representative genes of these pathways in the colon. We observed no effect of Lacto-Control or Lacto-hACE2 on the pro-inflammatory genes tested, including *Il-6, Il-10*, and *Il-17a*, and no effect of Lacto-Control or Lacto-hACE2 on the relative expression levels of the sodium transporter gene *Scnn1a* (Supplementary Fig. S1). However, as shown in Fig. 5, we observed significantly lower relative expression levels of *Glut1, Eno1, Ldha*, and *Pgam1* in the colon of Lacto-hACE2-treated female but not male *Ace2* KO rats.

4. Discussion

In this study, we investigated the effectiveness of engineered *Lactobacillus paracasei* bacteria to deliver an antihypertensive agent. Our results show that oral administration of Lacto-hACE2 notably lowered BP in the *Ace2* KO hypertensive rat. This beneficial effect was observed only in female rats, indicating a sex-specific reduction of BP. Further analysis revealed that the BP lowering effect in females was aligned with decreased colonic AngII levels, but not renal AngII. This study is the first to demonstrate that Lacto-hACE2 can lower BP in the female r*Ace2* KO rats.

The *Ace2* gene is located on the short arm of the X chromosome (https://www.ncbi.nlm.nih.gov/gene/302668) and has been linked to sex-specific hypertension due to its role in downregulating AngII [41, 42]. Since gut microbiota is an important regulator of hypertension [43] and colonic *Ace2* expression [44], we hypothesized that disrupting *Ace2* would lead to higher BP, and that administering beneficial gut microbiota (i.e. *Lactobacillus paracasei*) expressing ACE2 would prevent hypertension. To test this hypothesis, a novel *Ace2* CRISPR/Cas9 gene-edited rat model was generated and validated. We observed decreased renal and colonic ACE2 activities in *Ace2* KO rats, confirming

the successful disruption of ACE2. As expected, male and female *Ace2* KO rats showed elevated BP than NF rats. However, oral delivery of ACE2 via Lacto-hACE2 rescued high BP only in female rats, not male rats, and was contributed by a dip in DBP along with lowered heart rates (Supplementary Fig. S2). In humans, it has been reported that resting heart rate is positively associated with DBP [45]. Additionally, we compared the proximal colon ACE2 activity between Lacto-hACE2 treated males and females to see if females had any advantage in terms of human ACE2 expression compared to males that contributed to the sex-specific blood pressure downregulation. There was no significant difference in ACE2 expression between males and females in the colon (data not shown), however, this sex-specific BP downregulation was accompanied by specifically lower AngII levels and AngII-induced colonic glucose transport and glycolysis transcript expression in the female colon.

In recent years, there has been a growing interest in understanding sex-specific mechanisms of BP regulation. Studies have revealed sex differences in the RAS and its downstream effectors [46]. Females require higher doses of AngII to cause an increase in BP compared to males [47]. Ang1–7, a physiological protective response, is more effective in females [48,49]. Interestingly, clinical evidence shows a disparity in the prescription of anti-hypertensive therapies between males and females [50], with females less likely to be prescribed ACE inhibitors but more likely to be prescribed diuretics [51]. Taken together, these findings suggest that the activation arm of the RAS (AngII) is more important in males, while the protective arm (ACE2, Ang1–7) is more important in females. Therefore, the disruption of ACE2 resulted in more increase in BP in the female rats, and successful restoration of ACE2 rescued the high BP in the females. This could explain the sex-specific BP rescue effect observed in the *Ace2* KO rats treated with Lacto-hACE2.

Interestingly, the rescued high BP in the females was associated with reduced colonic AngII, rather than the renal AngII. This may be due to the fact ACE2 expressed by Lacto-hACE2 was mainly accumulated in the gut [24]. Ang II stimulates glucose transport via glucose transporter 1 (GLUT1) [38,39], and overexpression of GLUT1 has been observed in Dahl S rats [52]. Furthermore, AngII has been shown to shift energy consumption towards glucose utilization in ACE2-KO mice [53] and directly influences glycolysis [40]. In light of the significant changes in AngII levels observed in the proximal colon of Lacto-hACE2-treated female rats, we compared the *Glut1* expression and found a significantly lower colonic *Glut1* expression in the *Ace2* KO rats treated with Lacto-hACE2. Since AngII directly influences glycolysis [40], we examined the expression of colonic glycolysis-related genes and observed inhibited expression of several transcripts, including *Eno1, Ldha*, and *Pgam* in Lacto-hACE2-treated female rats. These three genes are critical glycolytic enzymes, with *Eno1* playing a crucial role in aerobic glycolysis by converting 2-phosphoglycerate into phosphoenolpyruvate [54], *Ldha* mediating the interconversion of pyruvate and lactate [55], and *Pgam* catalyzing the interconversion of 3-phosphoglycerate and 2-phosphoglycerate during glycolysis [56]. Collectively, our results suggest that Lacto-hACE2 lowered BP in female rats by targeting colonic AngII and AngII-induced glucose metabolism.

As one of the therapies targeting gut microbiota, probiotics is being investigated for hypertension [14,57]. Among them, *Lactobacillus* is known as a beneficial microbe that

decreases BP in both rats [58] and humans [59]. Guilian Yang, et al. [60] found the recombinant *Lactobacillus plantarum* expressing ACE inhibitory peptide could effectively treat hypertension in male rats. *Lactobacillus* was also engineered to treat diabetes. Most recently, Ram Prasad et al. [24,25] used the Lacto-hACE2 to treat diabetic retinopathy in type 1 diabetes in mice. However, to our knowledge, recombinant *Lactobacillus* expressing human ACE2 has not been tested for hypertension.

Our data here provided the rationale for microbiome-based medicine against hypertension by demonstrating the efficacy of Lacto-hACE2 to ameliorate hypertension in the newly generated *Ace2* KO rats.

5. Conclusions

The three main findings in this study can be summarized as: (i) the engineered commensal bacteria can effectively deliver antihypertensive biologics and reduce BP; (ii) *Lactobacillus paracasei* engineered to produce human ACE2 was the specific biologic that proved effective in hypertension; and (iii) the mechanism by which Lacto-ACE2 reduced BP in female rats could be due to decreased expression of colonic AngII and glucose metabolism related transcripts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Graphical abstract created with BioRender.com.

Data Availability

No data was used for the research described in the article.

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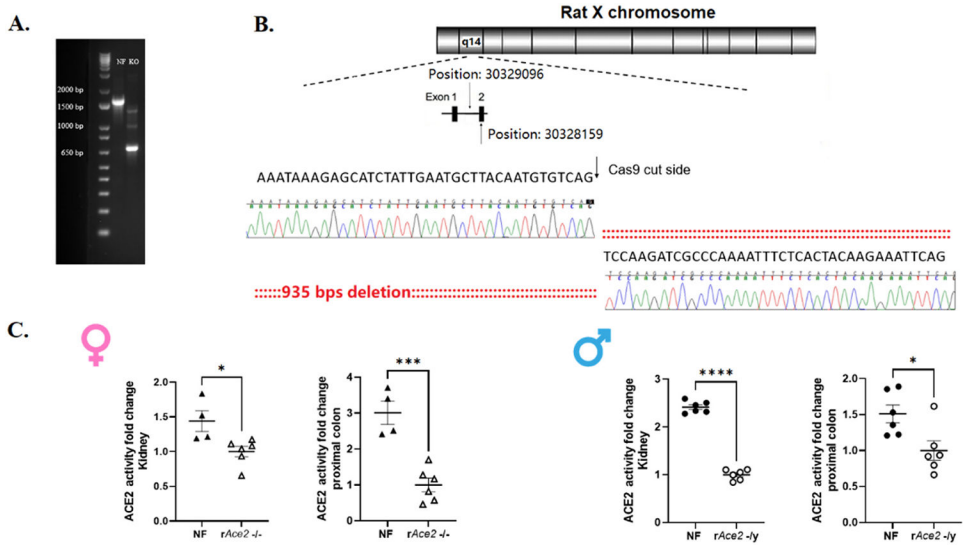

Fig. 1.

Screening and characterization of rats for CRISPR/Cas9-mediated deletion of the rat *Ace2* locus. (A) Amplification of genomic DNA from NF (left full band) and KO rat (right lower band). (B) *Ace2* KO rats contained a 935-basepair deletion within the *Ace2* locus. Gene ID: 302668 (https://www.ncbi.nlm.nih.gov/gene/302668). (C) ACE2 protein activity in the kidney and colon of male and female NF and KO rats. NF female, n = 4, r*Ace2*$^{-/-}$ female, n = 6; NF male, n = 6, r*Ace2*$^{-/y}$ male, n = 6. *Ace2*/ACE2, angiotensin converting enzyme 2; NF, non-founder, the control rats; r*Ace2*$^{-/-}$ female *Ace2* KO rats; r*Ace2*$^{-/y}$, male *Ace2* KO rats. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ (unpaired t test).

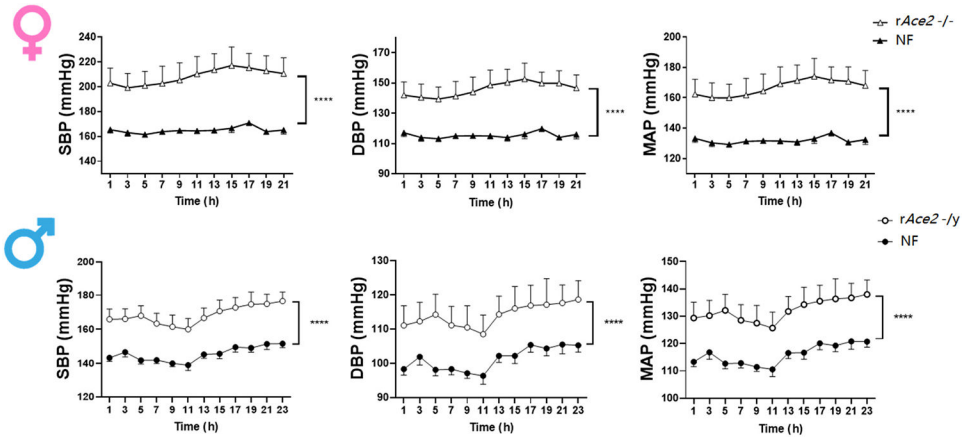

Fig. 2.

Systolic, diastolic, and mean arterial pressures in male and female r*Ace2* KO and NF rats. NF female, n = 7, r$Ace2^{-/-}$ female, n = 5; NF male, n = 7, r$Ace2^{-/y}$ male, n = 7. ****$p < 0.0001$ (two-way ANOVA followed by LSD test). SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean atrial pressure; NF, non-founder rats; r$Ace2^{-/-}$, female *Ace2* KO rats; r$Ace2^{-/y}$, male *Ace2* KO rats.

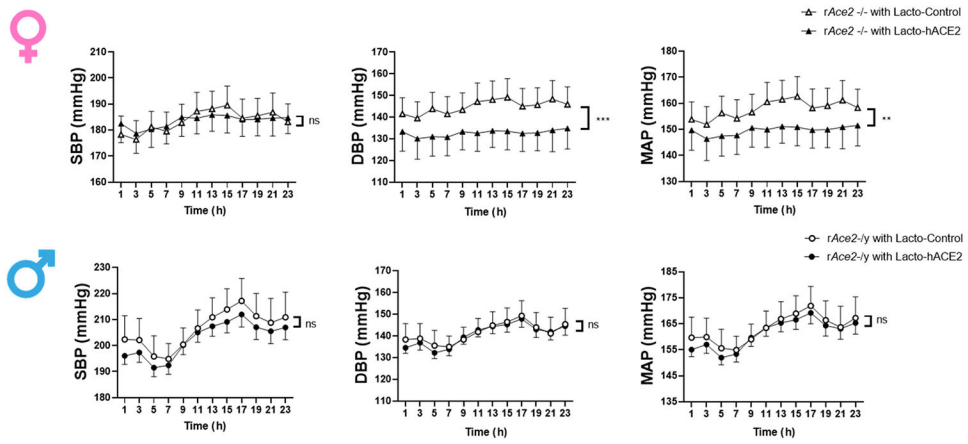

Fig. 3.

Systolic BP, diastolic BP and mean atrial pressure in male and female *Ace2* KO rats following treatment with Lacto-Control and Lacto-hACE2. Lacto-Control treated female, n = 8, Lacto-hACE2 treated female, n = 7; Lacto-Control treated male, n = 6, Lacto-hACE2 treated male, n = 8. $^{**}p < 0.01$; $^{***}p < 0.001$ (two-way ANOVA followed by LSD test; ns, not significant). SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; Lacto-Control, wild type of *Lactobacillus paracasei*; Lacto-hACE2, *Lactobacillus paracasei* expressing the human ACE2.

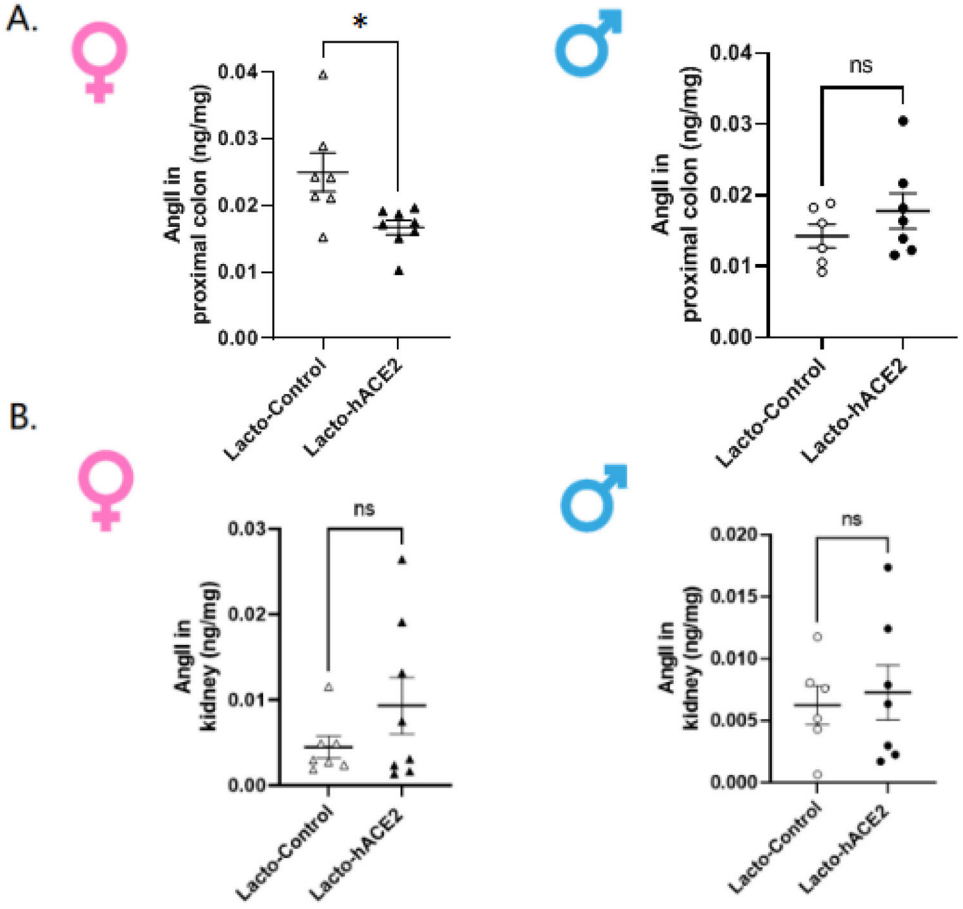

Fig. 4.

AngII protein levels in the proximal colon (A) and kidney (B) of *Ace2* KO rats treated with Lacto-Control and Lacto-hACE2. Lacto-Control treated female, n = 7, Lacto-hACE2 treated female, n = 8; Lacto-Control treated male, n = 6, Lacto-hACE2 treated male, n = 7. * $p < 0.05$ (unpaired t-test, ns, not significant). Lacto-Control, wild type *Lactobacillus paracasei*; Lacto-hACE2, *Lactobacillus paracasei* expressing the human ACE2.

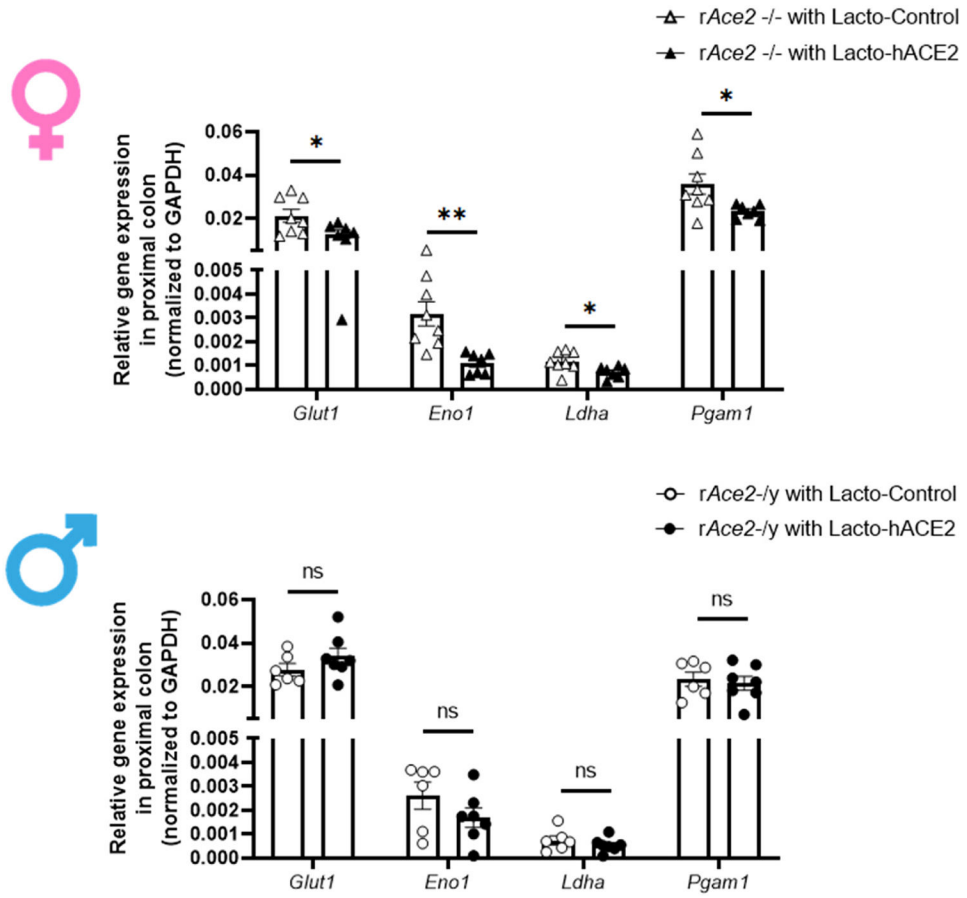

Fig. 5.

Relative expression levels of genes related to glucose transport and metabolism in the colon of male and female *Ace2* KO rats treated with Lacto-Control and Lacto-hACE2. Lacto-Control treated female, n = 8, Lacto-hACE2 treated female, n = 7. Lacto-Control treated male, n = 6, Lacto-hACE2 treated male, n = 7. *$p < 0.05$, **$p < 0.01$ (unpaired t-test). Lacto-Control, wild type *Lactobacillus paracasei*; Lacto-hACE2, *Lactobacillus paracasei* expressing the human ACE2.